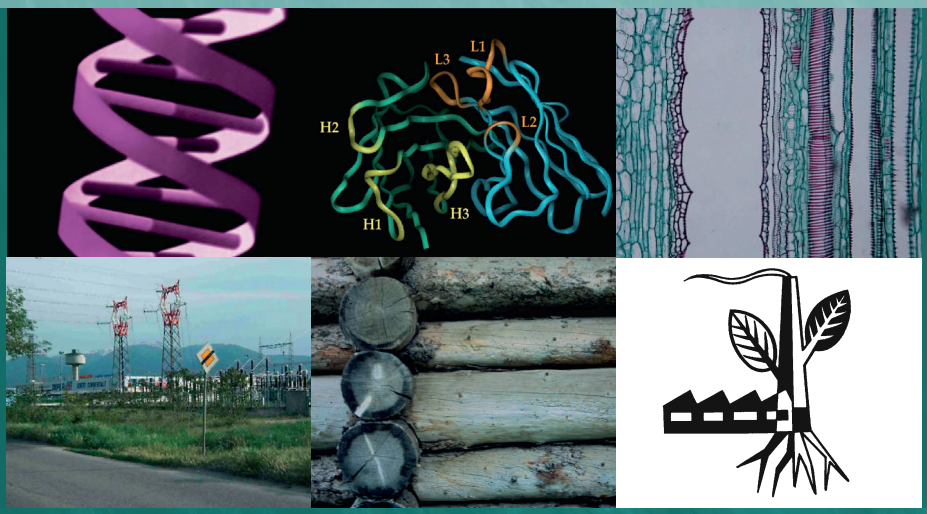
P. Ranalli
Editor

Improvement of Crop Plants for Industrial End Uses



Springer

IMPROVEMENT OF CROP PLANTS FOR INDUSTRIAL END USES

Improvement of Crop Plants for Industrial End Uses

Edited by

P. RANALLI
C.R.A. -ISCI, Bologna, Italy

Springer

A C.I.P. Catalogue record for this book is available from the Library of Congress.

ISBN-10 1-4020-5485-8 (HB)
ISBN-13 978-1-4020-5485-3 (HB)
ISBN-10 1-4020-5486-6 (e-book)
ISBN-13 978-1-4020-5486-0 (e-book)

Published by Springer,
P.O. Box 17, 3300 AA Dordrecht, The Netherlands.

www.springer.com

Printed on acid-free paper

TABLE OF CONTENTS

Preface

New uses are already being found for existing crops (e.g. bioethanol and biodiesel from oil crops) and "new" crops are being developed (e.g. willow/poplar for biomass production, and primrose for pharmaceutical uses). Advanced biotechnologies will enable breeders to produce a whole generation of new crops for specialist needs ("designer crops"), including raw materials for the energy, chemical and pharmaceutical industries.

In this book, the advances gained in different fields of research are reviewed. In particular, research carried out on the application of biotechnology in order to: i) produce new compounds by expressing foreign heterologous genes in specific parts of a plant, ii) or modify pathways to influence quality and/or yield of existing indigenous target molecules, iii) or bioprocess plant or organic waste stream into value-added products. The gained know how is basical to identify bottlenecks in the major production chains and to propose actions for moving these issues forward.

This book gives an overview of recent research and technological development in biotechnology and biobased products and addresses the following thematic priorities: *Metabolic engineering*. Work on gene sequencing (genomics) and cataloguing the proteins that genes express (proteomics) has reached relevant advances in recent years. Unfortunately, research on plant metabolic pathways attracted, during the 1990s, much lower levels of funding than genomics. Consequently, a paucity of knowledge in the area of plant metabolic processes is identified as the main scientific constraint to the rational development of bio-based products using biotechnology. *Plant breeding tools*. Conventional breeding can dramatically increase yields and improve biomaterial qualities. Moreover, genetic markers can now assist plant breeders by identifying lines with genes linked

to particular traits. However, genetic manipulation offers the greatest potential for producing raw materials in a volume and of a quality attractive to industry. It is also a tool for producing novel biomaterials which are unobtainable in plants by any other means. *Renewable biomass for energy generation.* Comparison of different provision chains with respect to bioethanol and biodiesel fuel production has been investigated. Cogeneration of biomass-based products as substitutes of fossils in the frame of greenhouse gas reduction strategies was explored. Vegetable oil-based solvents and lubrificants are already used in many industrial niche markets, but industry may soon be looking for a much greater range of plant-derived oils as alternatives to fossil-based oils. Biotechnology will play a key role in raising oil yields, fine-tuning metabolic processes to improve oil quality, and engineering novel oils for specific applications. *Fibres and composites*. Modified plants with improved fibre qualities could exploit new markets for biodegradable composites, textiles and fabrics. Biotechnology is also helping to improve the efficiency of wood and pulp processing by reducing the content of lignin and other polymers in woody plant tissue. Fibres from novel crops are replacing petroleum-based composite materials used by industry. *Biopharmaceuticals.* The plants have great potential for economical, large-scale biopharmaceutical production and they have been explored to produce vaccine components, such as antigens and/or adjuvants; in this field, plants can be used merely as "biofactories" for the massive production of the product of interest or, in addition, as a tool for vaccine delivery. The different plant-based production systems used to synthesize recombinant antibodies are discussed and the merits of plants compared with other platforms evaluated.

Conclusively, the new book provides concerns useful to promote an increase of the productivity of crops by using functional genomics (to understand the regulation of plant metabolism at molecular, cellular and whole plants) and the improvement of photosynthetic efficiency (to design new plants with enhanced raw materials percent and recovery).

The Chapters of this book have been written by experts from all around the world. Consequently, the book is expected to be of great interest for scientists, researchers, farmers, processors and retailers, but also for students, technocrats and planners interested in the progress made with the development of new industrial crops.

Paolo Ranalli

1

The use of functional genomics to understand components of plant metabolism and the regulation occurring at molecular, cellular and whole plant levels

Paolo Pesaresi[1,2]

[1]Parco Tecnologico Padano, Via Einstein, Loc. Cascina Codazza, 26900, Lodi, Italy

[2]Dipartimento di Produzione Vegetale, Università Statale di Milano, Via Celoria 2, 20133 Milano, Italy (e-mail: paolo.pesaresi@tecnoparco.org)

1. Introduction

The completion of the genome sequence of the small weed plant *Arabidopsis thaliana* (The Arabidopsis genome initiative 2000), and more recently of rice (Goff et al. 2002; Yu et al. 2002, 2005), has greatly changed the face of plant biology. Knowing the exact sequence and location of all the genes of a given organism is the first step towards understanding how all parts of a biological system work together. Information about the hypothesized function of an unknown gene may be deduced from its sequence homology to other genes of known function. However, genome sequencing projects have revealed the existence of a tremendous amount of biological diversity, with large proportion of genes sharing no homology to genes with known or hypothesized functions. In this respect functional genomics is the key approach to transforming quantity into quality (Borevitz and Ecker 2004; Holtfort et al. 2002). Functional genomics is a general approach toward understanding how the genes of an organism work

P. Ranalli (ed.), Improvement of Crop Plants for Industrial End Uses, 1–26.

together by assigning new functions to unknown genes. For efficient gene function analysis, researchers can choose from a multitude of different methods, most of them derived from genomic research performed on model organisms such as yeast, nematodes, flies and mice, not forgetting the technological spin-offs that were inspired by the human genome project. Arabidopsis populations, mutagenized by random insertion of T-DNA or transposon elements, have been generated with the aim to perform high-throughput reverse genetics studies and comprehensive forward genetics studies of the entire gene compendium (Alonso et al. 2003). Additionally, information about the spatial and temporal expression pattern of a gene can be gained from analysis of qualitative and quantitative changes of messenger RNAs, proteins, and metabolites. These techniques, able to simultaneously analyze large numbers of transcripts, proteins and chemical constituents, have led to the creation of new research fields within functional genomics, named transcriptomics, proteomics, and metabolomics. Each method has its inherent limitations and none of them alone is sufficient to assign a function to a gene of interest. However, the organization of the vast amount of data from the various approaches into central databases allows easy extraction and comparison of meaningful information.

This chapter has the aim to highlight the major approaches that makes up modern plant functional genomics and to describe how they add a new dimension to the comprehension of plant biology with particular emphasis to the model plant, *Arabidopsis thaliana*.

2. Plant genome sequences

The public effort to sequence the genome of the model flowering plant, *Arabidopsis thaliana*, was completed in December 2000 (The Arabidopsis genome initiative 2000), and it was the third complete genome of a higher eukaryote, after *Drosophila melanogaster*, and *Caenorhabditis elegans*. This tiny mustard plant, a common weed of the Brassicaceae family, had been chosen as the first reference plant to be sequenced, because it has several advantages over other species. Its nuclear genome is very small with 115 million base pairs (Mb) of euchromatin out of the estimated 125 Mb total, its generation time is very short, and it is genetically very well characterized. The total number of Arabidopsis genes revealed by the five chromosome sequences was initially estimated at 25,490 and later revised to 30,700 (version 5 annotation), resulting in 11,000-15,000 gene families, with about one quarter of genes believed to be plant specific. The entire *Arabidopsis* genome dataset has been stored and can be retrieved from

different user-friendly databases, including TAIR, TIGR, MIPS and NCBI (Table 1.1). In 2002, two groups released the second plant genome sequence, rice. A four time shotgun coverage of *Oryza sativa* ssp. *indica* covered 361 Mb of the estimated 466 Mb (Yu et al. 2002). *Oryza sativa* ssp *japonica* was sequenced to five times shotgun coverage (Goff et al. 2002) and resulted in 372 Mb of non-overlapping sequence from the 12 rice chromosomes and 55,890 genes were identified (version 4 annotation). More recently, improved whole genome shotgun sequences for the genomes of indica and japonica rice have been reported (Yu et al. 2005). Sequences and analysis details of rice genome are available at several databases including TIGR, and Rice Genome research Program (Table 1.1). The completely annotated reference genomes of Arabidopsis and rice certainly serve as a starting point for the large-scale functional analysis of other plant genomes. Indeed, many other plant species have entered the genomics era. In particular, the Joint Genome Institute (JGI) has essentially finished a deep draft genome sequence of the model tree *Populus trichocarpa* (cottonwood). Ten-times shotgun coverage, amounting to 5.5 Gb, is now available for download and BLAST searches at the JGI database (Table 1.1). Moreover, approximately 80,000 ESTs have been sequenced and will certainly be helpful for gene annotation. *Medicago truncatula* has been chosen as the model legume plant for genomics studies. The complete genome of the first legume will certainly speed up the comprehension of the molecular mechanisms responsible for the conversion of molecular nitrogen into usable organic forms (legume/*Rhizobium* symbiosis). Another crop plant that has been used as a model for decades is *Lycopersicon esculentum* (tomato). The genome is about 900 Mb and its sequencing is a priority according to the National Plant Genomics Initiative. At the moment more than 150,000 *Lycopersicon esculentum* ESTs, stored at Cornell University (Table 1.1), are available. Additionally, the sequencing of all twelve chromosomes has been initiated.

3. Genome-wide insertional mutagenesis

One of the most significant findings revealed through analysis of plant genomes is the large number of genes for which no function is known or can be predicted. An essential tool for the functional analysis of these completely sequenced genomes is the ability to create loss-of-function mutations for all the genes (Borevitz and Ecker 2004). A knockout line can provide a crucial second allele when only a single EMS allele is available. Here observation of similar phenotypes in both alleles makes sure that the correct gene was identified. The null genetic background is suitable for

transgenic studies that investigate altered expression patterns or test altered proteins. Often redundancy can confound genetic studies by masking phenotypes. This problem can be dealt with by creating double, triple, or greater knockout mutations among multiple gene family members. Although, targeted gene replacement via homologous recombination is extremely facile in yeast, its efficiency in plants does not yet allow for the creation of a set of genome-wide gene disruptions (Gong and Rong 2003; Parinov and Sundaresan 2000). Additionally, gene silencing via the RNA interference (RNAi) method has several drawbacks, including the lack of stable heritability of a phenotype, variable levels of residual gene activity, and the inability to simultaneously silence several unrelated genes (Hannon 2002). Because of these disadvantages, the random insertions of T-DNA or transposon elements has become the strategy of choice to generate loss-of-function Arabidopsis populations. In particular, Alonso and colleagues (2003) have generated about 150,000 transformed plants carrying one or more T-DNA elements. After sequencing analysis, about 88,000 T-DNA integration sites were identified, resulting in the disruption of 21,799 genes, about 74% of the Arabidopsis gene repertoire (T-DNAexpress, Table 1.1). Several other studies have created sequence-index collections of knockout mutations, including the French program Inra/Genoplante (Samson et al. 2002), the German project GABI-Kat (Li et al. 2003), the Japanese group in RIKEN (Kuromori et al. 2004), and the Cold Spring Harbor Laboratory (Table 1.1). Nowadays, there are more than 360,000 Arabidopsis flanking sequences that hit more than 90% of the currently known genes. Nearly all sequences can be searched at the signal T-DNAexpress website and corresponding seeds can be ordered both at the European Arabidopsis stock center and at the Arabidopsis Biological Resource Center (ABRC), located at the Ohio State University (Table 1.1).

Table 1.1. Websites relevant to plant functional genomics.

Plant genomic databases	
The Arabidopsis Information Resource (TAIR)	http://www.arabidopsis.org/
The Institute for Genomics Research (TIGR)	http://www.tigr.org/plantProjects.shtml
Munich Information center for Protein Sequences (MIPS)	http://mips.gsf.de/projects/plants
National Center for Biotechnology Information (NCBI)	http://www.ncbi.nlm.nih.gov/
Rice Genome research Program (RGP)	http://rgp.dna.affrc.go.jp/
Joint Genome Institute (JGI)	http://www.jgi.doe.gov/
Tomato Expression Database	http://ted.bti.cornell.edu/

Table 1.1. Websites relevant to plant functional genomics.

T-DNAexpress	http://signal.salk.edu/cgi-bin/tdnaexpress
Inra/Genoplante	http://urgv.evry.inra.fr/projects/FLAGdb++/HTML/index.shtml
GABI-Kat	http://www.gabi-kat.de/
RIKEN	http://rarge.gsc.riken.jp/dsmutant/index.pl
Cold Spring Harbor Laboratory	http://genetrap.cshl.org/
European Arabidopsis Stock Center	http://arabidopsis.info/
Arabidopsis Biological resource center (ABRC)	http://www.arabidopsis.org/abrc/
Microarray databases	
NASCArrays	http://affymetrix.arabidopsis.info/narrays/experimentbrowse.pl
ArrayExpress	http://www.ebi.ac.uk/arrayexpress/
Gene Expression Omnibus (GEO)	http://www.ncbi.nlm.nih.gov/geo/
GENEVESTIGATOR	https://www.genevestigator.ethz.ch/
Proteomics databases	
Swiss-prot	http://www.expasy.org/sprot/ppap/
Plastid Proteome Database (PPDB)	http://ppdb.tc.cornell.edu/
Arabidopsis Mitochondrial Protein Database (AMPD)	http://www.plantenergy.uwa.edu.au/applications/ampdb/index.html
Aramennon database	http://aramemnon.botanik.uni-koeln.de/
Protein-GFP fusions	http://deepgreen.stanford.edu/
Subcellular location database Arabidopsis (SUBA)	http://www.plantenergy.uwa.edu.au/applications/suba/index.php
Plant Phosphorylation database (PlantP)	http://PlantsP.sdsc.edu.
Metabolomics databases and Computational tools	
Golm Metabolome Database (GMD)	http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/gmd.html
Kyoto Encyclopedia of Genes and Genomes (KEGG)	http://www.genome.jp/kegg/
BRaunschweig ENzyme DAtabase (BRENDA)	http://www.brenda.uni-koeln.de/
AraCyc	http://www.arabidopsis.org/tools/aracyc
MAPMAN	http://gabi.rzpd.de/projects/MapMan/
MetNet	http://metnet.vrac.iastate.edu/

4. Forward and reverse genetics-essential steps towards understanding gene function

The classical or "forward genetics" approach to gene function analysis aims to identify the sequence change that underlies a specific mutant phenotype (Ostergaard and Yanofsky 2004). In the recent past, the starting point involved mutagenesis of a large number of wild-type seeds by treatment with chemical reagents or irradiation. Such treatments typically introduced single nucleotide changes or small deletions in the genome, resulting in mutant collections to be screened for the phenotype of interest (Feldmann et al. 1994; Greene et al. 2003). Even today, there are still good reasons to screen for mutants in chemically mutagenised populations, including the possibility to have an allelic series, allowing for strong, intermediate and weak alleles of genes that otherwise would produce lethal phenotypes when inactivated. Such conventional forward approach is, however, inefficient when it comes to performing high-throughput functional genomics analyses. Indeed, despite the existence of genetic and physical maps, the map-based cloning still remains a laborious approach (Peters et al. 2003). Screening of populations generated by random insertion mutagenesis, in contrast, has the advantage to allow the isolation of the disrupted gene, causing the observed phenotype, in a much more direct way. Indeed, the precise chromosomal location of individual inserts can be easily obtained by PCR-based methodologies, such as thermal asymmetric interlaced PCR (TAIL; Liu and Whittier 1995), and amplification of insertion mutagenised sites (AIMS; Frey et al. 1998).

Whereas forward genetics starts with the mutant and then leads to the gene, reverse genetics starts with the gene of interest and ends with the corresponding mutant. As mentioned above, databases containing sequence information of Arabidopsis DNA stretches flanking the insertion sites are available and they can be easily searched for the knockout of interest. This approach is particularly useful to verify the role of genes whose functions are already known in other species (Bellafiore et al. 2005). However, loss-of-function lines not always exhibit an evident phenotype. Meinke et al. (2003) estimate that of all the predicted Arabidopsis genes only about 10% of them are expected to result in a detectable loss-of-function phenotype. Indeed, about 65% of Arabidopsis genes appear to be members of families with two or more members, and functional redundancy among closely related genes often obscures their phenotypes. To circumvent this problem, reverse genetics allows mutations in all members of a gene family to be identified. Indeed, simple crosses can be performed to combine mutations in closely related genes, resulting in phenotypes that

would otherwise remain hidden. In addition, some phenotypic characteristics may be hard to detect unless the mutated gene is studied in a certain mutant background that more clearly reveals its loss-of-function phenotype (Roeder et al. 2003). Assessing a possible phenotype may also depend on the assay conditions. For instance, mutations in genes that are involved in stress responses may only display a detectable phenotype when subjected to certain environmental challenges. Reverse genetics largely facilitates these kinds of studies by allowing scientists to make qualified guesses on which combination of mutations would give rise to phenotypic changes.

5. Transcriptomics - depicting the expression level of genes

Obtaining mutations in genes of interest is an important and necessary step, however is just one of many powerful tolls that are needed to understand how the function of a gene is carried out. Genome-wide analysis of gene expression can be used as a powerful methodology to provide further information on gene activity. In this sense the development of full genome oligonucleotide-based microarrays, such as the ATH1 by Affymetrix, which represents approximately 23,750 Arabidopsis genes, allow to monitor at the same time the abundance of thousands of mRNA molecules (Redman et al. 2004). The classical experiment involves the simultaneous measurement of the relative concentration of a given transcript in two different samples by competitive two-colour hybridization. Most experiments use reverse transcription for labeling the cDNAs of both samples. cDNA molecules are labeled with different fluorophores (e.g. fluorescein, Cy5, Cy3). After hybridization to the arrayed target molecules the relative measure of gene expression for each gene analyzed is determined with the help of a high-resolution laser scanning device. Scanning is conducted using two different wavelengths, giving a quantitative fluorescence image for the two different probe populations (Lockhart and Winzeler 2000). Expression profiling experiments employing microarrays can be used to address diverse biological problems. For instance, by monitoring different tissues and developmental stages, an atlas can be created that describes the expression pattern of every gene in the genome (Borevitz and Ecker 2004). Knowledge of the timing and expression pattern of genes allows potential network to be created. The biological function of unknown genes in such a network can be inferred under the assumption that genes expressed similarly will be involved in a similar process (Brown and Botstein 1999;

Oliver 2000). Additionally, by looking at the changing expression patterns in response to abiotic or biotic stresses, one can identify the complete set of genes that is involved in a certain biological process. Defining such sets of genes, which are co-regulated under defined conditions, also allows for the identification of "marker genes" that are diagnostic for certain developmental or environmental processes (Holtorf et al. 2002). Alternatively, comparison of the expression profiles of wild-type plants to mutant lacking the activity of a stress- or development-induced transcription factor gene allows the identification of genes modulated by that transcription factor. Since the microarray-based expression profile technology has been established, thousands of arrays have been processed, of which a significant number are publicly available through services and repositories (Table 1.1). The Nottingham Arabidopsis Stock Centre Transcriptomics Service, NASCArrays, is one of them. Currently the database contains 40 experiments made up of about 400 GeneChips from Affymetrix system, but the number is increasing rapidly (Craigon et al. 2004). Other repositories include the ArrayExpress at the European Bioinformatics Institute (Brazma et al. 2003), and the Gene Expression Omnibus (GEO; Edgar et al. 2002) at the National Center for Biotechnology Information. However, the combination of multiple datasets still raises a number of questions concerning their compatibility, in particular when comparing data from different platforms. To overcome this problem, GENEVESTIGATOR (Table 1.1), a database and Web-browser data mining interface, has been created with the peculiarity to contain exclusively data from Affymetrix GeneChip (Zimmermann et al. 2004, 2005). Although data from different experiments may not be pooled for a rigorous expression profiling analysis, one can assume that the large scale combination and analysis of expression data from a single platform like the Affymetrix system allows the identification of biologically meaningful expression patterns of individual genes. Currently, the database covers more than 150 experiments, 28 plant organs and ten growth stages, in addition to responses to 68 environmental factors and to more than 100 genetic modifications. The dataset can be presented in the context of plant development, plant organ, environmental conditions, and mutated genetic backgrounds, both for individual genes or for families of genes, thereby answering questions such as "which other genes are coexpressed with the one of interest?", "in which organ or tissue is expressed the gene of interest?", "in which section of the life cycle the gene of interest is expressed?", or "which environmental stimulus induces the expression of the gene of interest?" The resulting answers can be used to confirm previous hypothesis or generate new hypotheses about gene expression network structures and genetic regulatory networks, resulting in the design

of more precise and targeted experiments aimed to gene function discovery.

6. Proteomics – protein compendium and interacting partners

It is mostly proteins that carry out cellular functions, therefore, for a comprehensive understanding of biological functions, the proteome of organelles, cells, and tissues has to be systematically characterised. The promise of proteomics is the precise definition of the function of every protein, and how that function changes in different environmental or developmental conditions, with different modification states of the protein, and with different interacting partners (Roberts 2002). The sequencing of the Arabidopsis and rice genomes has greatly aided the scope for the discovery and exploitation of the plant proteome (Swiss-prot; Table 1.1). Large-scale transcript analysis now allows high-fidelity assessments of the tissue and developmental profiles of probable Arabidopsis proteomes, albeit with the caveat that differences in transcript abundance underlie differences in protein abundance. However, even these experimental approaches largely neglect the cellular compartmentalization of plant cells. The products of thousands of genes in plants are efficiently targeted to particular parts of the cell by elaborate targeting machinery that uses targeting information within the amino acid sequence of proteins (Heazlewood et al. 2005). Identifying protein locations within the plant cell is thus an important step toward a broader understanding of cellular function as a whole, and provides vital assistance in identifying the role of the many proteins currently ascribed to unknown function in plant genome databases. Several routes can be taken to place this cellular compartmentalization perspective on plant genomic data. The use of bioinformatics targeting algorithms to predict where gene products will be located is a simple, low-cost, and rapid way to tackle this issue. An array of such programs, able to predict protein localization into the nucleus, mitochondrion, plastid, peroxisome, and endoplastic reticulum, exists. However, a significant limitation of this approach is the lack of prediction capabilities for compartments, such as the Golgi, vacuole, and plasma membrane. A first prediction aimed to identify nuclear-encoded proteins targeted to chloroplasts was performed by Abdallah et al. (2000). These authors analysed the, at that time, partially sequenced genome of *A. thaliana*, employing the neural network-based program ChloroP (Emanuelsson et al. 1999), and extrapolated a total number of around 2,200 proteins having a chloroplast transit peptide (cTP). Based on

the ChloroP algorithm, the TargetP program was, subsequently, developed by Emanuelsson et al. (2000), and it was estimated that more than 3,000 genes of the nuclear genome of *A. thaliana* encode for proteins featuring a cTP. More recently, the accuracy of the four most-widely used cTP predictors, iPSORT (Bannai et al. 2002), TargetP (Emanuelsson et al. 2000), PCLR (Schein et al. 2001) and Predotar (http://urgi.infobiogen.fr/predotar/predotar.html), was re-evaluated on a test set of 2,450 proteins with known subcellular location, and was found to be substantially lower than previously reported (Richly and Leister 2004). A combination of cTP predictors resulted to be superior to any one of the predictors alone and was employed to estimate that around 2,000 different cTP-proteins should exist in *A. thaliana*. Clearly, the large discrepancies among different type of predictors highlights the need of direct experimental approaches to better identify organelle and compartment proteomes. The strategies most commonly used involve cellular fractionation, centrifugation-based purification of an organelle, or cellular compartment and mass spectrometry (MS) to identify peptides (Millar 2004). A series of reports has provided in-depth analyses of chloroplast proteome. Norbert Rolland, Jacques Joyard and colleagues have analyzed a mix of inner and outer envelope membrane proteins of chloroplast from spinach and *A. thaliana* (Ferro et al. 2002; Seigneurin-Berny et al. 1999). Several known, as well as novel, membrane proteins were identified. In their latest, more extensive study with mixed *A. thaliana* chloroplast envelope membranes, more than 100 proteins were identified (Ferro et al. 2003). Almost one third of the identified proteins was reported to have no known function, whereas more than 50% were very likely to be associated with the chloroplast envelope, based on their postulated function or because they were already known envelope proteins. Wolfgang Schröder, Thomas Kieselbach and colleagues also analyzed the lumenal proteome of *A. thaliana* and spinach (Kieselbach et al. 1998; Schubert et al. 2002), resulting in the identification of thirty-six proteins. Similarly, several groups have contributed to the investigation of the mitochondrial proteome (Eubel et al. 2003; Herald et al. 2003; Kruft et al. 2001; Millar et al. 2001; Werhahn and Braun 2002). Recently, a large analysis using non-gel proteomic approaches based on liquid chromatography and tandem MS (LC MS-MS) has provided a set of more than 400 non-redundant proteins from Arabidopsis mitochondria (Heazlewood et al. 2004). The proteome of nuclei, vacuoles, and peroxisomes has also received attention in recent reports (Carter et al. 2004; Fukao et al. 2002; Pendle et al. 2005). A series of studies has also identified proteins among the other intracellular membrane systems, including plasma membranes, Golgi, and endoplasmic reticulum (Alexandersson et al. 2004; Prime et al. 2000; Santoni et al. 1999).

A complementary approach to MS in identifying protein location is the expression and visualization of fluorescence proteins (FPs) attached to proteins of interest. A range of differently coloured fluorescent proteins have been used, including green fluorescent protein (GFP), red fluorescent protein (RFP), yellow fluorescent protein (YFP), and cyan fluorescent protein (CFP), with GFP being the dominant choice. Increasingly referred to as clone-based proteomics, single–protein studies, medium throughput approaches, and even high-throughput GFP screening of protein locations using this technique are currently under way in *Arabidopsis* (Cutler et al. 2000; Koroleva et al. 2005). Many hundreds of proteins have been visualised in this manner to date and form an important dataset for determining subcellular localization. Concerning *Arabidopsis*, it has been calculated that all the different approaches have resulted in the localization of 4,418 proteins, representing approximately 15% of the whole predicted proteome. All these information distributed in a large set of databases (Table 1.1) have been recently collected in the Subcellular location database for Arabidopsis proteins (SUBA; Heazlewood et al. 2005), which provides an integrated understanding of protein localization, encompassing the plastid, mitochondrion, peroxisome, nucleus, plasma membrane, endoplasmic reticulum, vacuole, Golgi, cytoskeleton structures, and cytosol. Of course, the subcellular localization data alone are not sufficient to determine the function of the protein of interest, however they represent an additive value towards the determination of gene function.

Another important aspect of proteomics concerns the characterization of covalent processing events, such as proteolytic cleavages and/or addition of modifying groups to one or more amino acids, responsible to change the properties of a protein (Huber and Hardin 2004; Mann and Jensen 2003). Far from being mere “decorations”, post-translation modifications of a protein can, indeed, determine its activity state, localization, turnover, and interactions with other proteins. Despite the great importance of these modifications for biological function, their study on a large scale has been hampered by a lack of suitable technologies. Indeed, proteomics has been very successful in identifying proteins in complexes and organelles since only few peptides are needed for protein identification, but they are not enough for complete primary-structure determination. A central consideration in the characterization of the modifications is the need for as large an amount of the protein as possible. Protein modifications are typically not homogenous, and a single gene can give rise to a bewildering number of gene products as a result of different modifications. Up to now most of the protein modifications have been analysed on a one-by-one basis and in many cases by using recombinant expressed proteins, but the real promise of proteomics is to asses systematically the modifications of large number

of proteins (Jensen 2000). Recent technological developments has made it increasingly feasible to directly analyse very complex peptide mixtures by LC MS-MS, and a single chromatographic run can result in the identification of hundreds of peptides, increasing substantially the chance of finding modified peptides. Moreover, the complexity of peptide mixtures can be reduced by affinity chromatography. For example, phosphopetides can be captured selectively through their negatively charged phosphogroup on immobilized metal affinity columns (IMAC; Nuhse et al. 2003). Recently, this technique has been used for a comprehensive analysis of phosphorylated membrane proteins in *Arabidopsis*, resulting in the identification of more than 300 phosphorylation sites (Nuhse et al. 2004). This analysis has yielded general principles for predicting other phosphorylation sites in plants and provided indications of specificity determinants for responsible kinases. In addition, more than 50 sites were mapped on receptor-like kinases and revealed an unexpected complexity of regulation. All the data have been collected in a new searchable database for plant phosphorylation sites (PlantP; Table 1.1), resulting in the first database on protein post-translational modifications, similar to the genomics, transcriptomics, and proteomics databases in existence today. Once post-translational modification analyses could be routinely done at proteomics level, the identification of more and more modification sites will dramatically increase, resulting in the development and tuning of algorithms aimed to the prediction of modification sites and to the functional interpretation of post-translational modifications.

The "omics" technologies has made clear that a discrete biological function can only rarely be attributed to an individual molecule. Instead, most biological characteristics arise from complex interactions between the cell constituents, such as proteins, DNA, RNA and small molecules. Therefore a key challenge for biology in the post-genomics era is to understand the structure and the dynamics of the complex intercellular web of interactions that contribute to the structure and function of a living cell. A prolific genome-wide approach to study protein-protein interactions is the yeast two-hybrid assay (Fields and Song 1989). This method constructs an artificial transcription factor, fusing the DNA-binding domain with the first query protein and a transcriptional activation-domain to the second protein or to an expression library. When two proteins bind, a reporter gene is actively transcribed. Thousands of protein interactions have been discovered with this strategy in yeast, *Caenorhabditis elegans*, and *Drosophila melanogaster* and recently this approach has been adopted also for *Arabidopsis* (Hackbush et al. 2005). In particular, the Uhrig group has investigated the interaction network of 3-aa loop-extension (TALE) homeodomain proteins. A combination of cDNA-library screenings and an

all-against-all pair wise interaction test revealed the formation of a complex array of homo- and hetero-dimers within the TALE family. In addition, a previously unrecognised plant-specific protein family denominated *Arabidopsis thaliana* ovate family proteins (AtOFPs) was involved in the highly interconnected TALE interaction network. An alternative technique under development for global discovery of protein-protein interactions is "fluorescence resonance energy transfer" (FRET) microscopy (Wouters et al. 2001). Fluorophores within about 60 Å of one another transfer energy, and tagging different fluorophores to a pair of proteins will generate observable resonant phenomena when the pair binds. The broad applicability of this method is not yet clear, but FRET has been successful in specific trials. High-throughput screens are currently in design. This system potentially has the great advantage of reporting both the localization and timing of protein interactions, *in vivo* and in response to experimental conditions or perturbations (Carter 2005). Array technology is another candidate for direct detection of protein-protein interactions. Taking DNA microarrays as a model, the aim is to construct a chip onto which an entire proteome is spotted (Schweitzer et al. 2003; Smith et al. 2005). This protein array would facilitate global screens not only for protein-protein interactions, but also for protein-DNA interactions. Indeed, the latter approach will be extremely useful to reveal the main actors, such as transcription factors and promoter *cis*-regulatory elements, responsible of the complex gene expression regulation mechanisms.

7. Metabolomics – comprehensive non-biased analysis of metabolites

Transcriptomics and proteomics certainly contribute to the description of phenotypes and discover of gene function, however, it is essential that phenotypic effects be described as explicitly as possible. To this aim, metabolites, regarded as the ultimate gene products, represent the direct link between genes and phenotypes. The plant kingdom is able to produce an astonishing wealth of metabolites, ranging from 90,000 to 200,000, both from primary and secondary metabolism (Fiehn 2002). Additionally, metabolites have a much greater variability in the order of atoms and subgroups with respect to the 4-letter code of genes and transcripts and the 20-letter code of proteins, making their characterization extremely demanding in terms of technologies. Accordingly, different analytical approaches have been designed in order to address specific questions. In particular, to directly study the primary effect of a genetic alteration, an analysis can be

constraint to the specific substrate and/or the direct product of the corresponding encoded protein. This strategy is called "targeted analysis" and is mainly used for screening purposes. Alternatively, the effects of biotic or abiotic stresses can be monitored on a selected number of predefined metabolites or pathways, by using the "metabolite profiling" strategy. However, quite frequently, genetic or environmental repercussions are not limited to one biological pathway. Indeed, the metabolite levels of unrelated pathways may be altered due to pleiotropic effects. In order to understand these effects, a comprehensive analysis in which all metabolites of a biological system are identified and quantified is needed. Such an approach has been called, "metabolomics". Metabolomics approaches must aim at avoiding exclusion of any metabolite by using well conceived sample preparation procedures and analytical techniques (Bhalla et al. 2005). To asses the enormous diversity of structurally complex chemical compounds, various approaches have been initiated in the last decade largely due to the tremendous advances in the instrumentation and data handling capabilities (Fukusaki and Kobayashi 2005). In particular, advances in metabolomics analysis owe primarily to improvements in the MS technology that has resulted in formats that are more user-friendly and amenable to biologists. Additionally, combination of mass spectrometry with in-line gas or liquid chromatography (GC-MS and HPLC-MS) has increased the efficiency of separation and identification of molecules. Nuclear Magnetic Resonance (NMR) is another potential very useful technique to be used in metabolomics, since in principle any chemical species that contains protons gives rise to signals. Indeed, NMR is often used for metabolite fingerprinting, where the aim is to look for compositional similarities and explore the overall natural variability (Bligny and Douce 2001; Raamsdonk et al. 2001). Recently, a new technique has been introduced, called Fourier Transform Ion Cyclotron Mass Spectrometry (FT-MS), that allows the study of phenotypic changes associated with metabolism. FT-MS is, indeed, suitable for rapid screening of similarities and dissimilarities in large collections of biological samples, such as plant mutant populations. Separation of the metabolites is achieved solely by ultra-high mass resolution. Identification of the putative metabolite or class of metabolites to which it belongs can then be obtained by determining the elemental composition of the metabolite based upon the accurate mass determination (Brown et al. 2005).

Similarly to genome, transcriptome and proteome fields, databases storing the flood of data arising from metabolomics analyses are becoming available. The Golm Metabolome Database (Kopka et al. 2005) is the first database that provides public access to custom mass spectral libraries, metabolite profiling experiments, as well as additional information and tools, e.g. with regard to methods, spectral information or compounds (Table 1.1).

Likewise transcriptomics, the primary objective of metabolomics analysis is to associate the relative changes in quantitative metabolite levels with functional assignments. To this aim, different pattern recognition methods such as hierarchical cluster analysis and principal component analysis can be applied to calculate an individual metabolite profile and compare it to other metabolite profiles. Profiles of samples which group into a defined cluster can then be used to define a metabolic phenotype. Once the existence of clusters within the samples is assured, classical statistics such as Student' s *t* test or multiple analysis of variance (MANOVA) can be applied in order to find statistically significant differences of metabolite levels between the clusters (Fiehn 2002). As with other functional genomics approaches, the interpretation of results may become problematic because of the sheer mass of data generated. The fact that biochemical pathways make up highly regulated networks adds to the complexity of the analysis. A way to interpret the data is to intercalate biochemical pathways, whether or not the alterations of metabolite levels or clustering results can be understood by known aspects of enzymatic regulation. With this respect, the Kyoto Encyclopedia of Genes and Genomes (KEGG; Ogata et al. 1999), represents a valuable tool (Table 1.1). In particular, KEGG is a knowledge base for systemic analysis of genes functions in terms of networks of genes and molecules. The major component of KEGG is the pathway database that consists of graphical diagrams of biochemical pathways and some of the known regulatory pathways. There are about 90 reference maps for the metabolic pathways that are manually drawn and continuously updated accordingly to biochemical evidences. In addition to the data collection efforts, KEGG provides various computational tools, such as for reconstructing biochemical pathways from the complete genome sequence and for predicting gene regulatory networks from the gene expression profiles. BRENDA is another database (BRaunschweig ENzyme Database; Schomburg et al. 2004) where a comprehensive collection of enzyme and metabolic information is collected. The database contains data from at least 83,000 different enzymes from 9800 different organisms, classified in approximately 4200 EC numbers. BRENDA includes biochemical and molecular information on classification and nomenclature, reaction and specificity, functional parameters, occurrence, enzyme structure, application, engineering, stability, disease, isolation and preparation, links and literature references (Table 1.1).

A number of different applications of metabolomics analyses can be imagined. For instance, metabolomics is being increasingly used for understanding the cellular phenotypes in response to various types of abiotic

or biotic stresses. In one recent study of sulphur deficiency response, general metabolic readjustment was found (Nikiforova et al. 2005). Mutual influences were found between sulphur assimilation, nitrogen imbalance, lipid breakdown, purine metabolism, and enhanced photorespiration. A general reduction of metabolic activity was seen under conditions of depleted sulphur supply. Metabolomics has also been applied to the case of cold stress response (Cook et al. 2004). In particular, a total of 325 metabolites were up-regulated in cold-treated Arabidopsis plants. Also in this case an extensive reconfiguration of several metabolic pathways could be observed. Concerning biotic stresses, Kant et al. (2004) investigated defence responses in tomato plants after infection with spider mites. Although the spider mites had caused little visible damage to the leaves after the first day of infection, they had already induced direct defense responses. For example, proteinase inhibitor activity had doubled. Moreover, at the fourth day after infection, a significant increase in the emission of volatile terpenoids could be observed. Alternatively, metabolomics data can be used to reveal the phenotype of silent mutations. Indeed, the intercellular concentrations of metabolites can reveal the site of action in the metabolic network of a disrupted gene. Moreover, metabolomics might be the ideal tool to investigate the substrate specificity of the several enzyme isoforms and, certainly, has a deep impact in prediction of novel metabolic pathways and in the description of cellular networks *in vivo*.

8. Naturally occurring genetic variation

Genetic variation found in wild strains is probably the most important basic resource for plant biology. In *Arabidopsis* genetic variation has been identified for many traits mainly by direct analysis and comparison of accessions. This evaluation is facilitated by the large collection of more than 300 different accessions collected worldwide, which are publicly available in the stock centers. In particular, genetic variation has been found for resistances to biotic factors such as bacteria, fungi, viruses, insects, and mammals (Koornneef et al. 2004). Variation for disease resistance genes is large and involves pathogens (Holub 2001) and many variants of one of the approximately 200 types of plant disease resistance genes of the so-called NBS-LRR classes (Meyers et al. 2003). Large variation has been also reported for tolerance to abiotic stresses and for developmental, physiological and biochemical traits (Koornneef et al. 2004). The study of genetic variation, certainly, is extremely useful to the identification of gene function. Indeed, despite the fact that mutant approaches have been very

powerful for functional analysis, often the definition of gene function is hampered by the genetic background of the analysed accession. In fact, the sort of mutant phenotypes that can be identified depends on the wild-type genotype. For instance, mutant phenotypes of genes for which the wild-type accession carries a natural null allele or a weak allele might not be detected. Examples of loss of function and probably null alleles present in accessions are quite common, as indicated by the 111 Columbia genes found to be partially or completely deleted in the *Ler* accession (Borevitz et al. 2003). Additionally, most variation among accessions is of a quantitative nature due to the effects of allelic variation at several loci, which combined with the environmental effect, determines a continuous phenotypic distribution of the trait in segregating populations (Quantitative Trait Locus, QTL). The analysis or mapping of QTLs, implies the identification of loci, the relative additive effects, the mode of action of each QTL (dominance effects) and, therefore the contribution of genetic interaction between loci, making it relevant to the comprehension of plant networks. To date, fine QTL mapping has been performed on a limited number of plant processes, including flowering time. Indeed, the timing of flowering transition is genetically differentiated among natural populations of *Arabidopsis*, as shown by the large genetic variation observed for this trait among Arabidopsis accessions. Currently, 14 different QTLs, accounting for flowering time differences among Arabidopsis accessions, have been identified. Among them, the strong effect loci *FRIGIDA* (*FRI*) and *Flowering Locus C* (*FLC*) (Johanson et al. 2000; Michaels and Amasino 1999). The isolation of these loci has led to the identification of two novel genes involved in the regulation of flowering, that could not be identified by mutant analyses because the common laboratory early flowering strains carry loss of function alleles at the corresponding loci. The *FRI* locus encodes a protein with no significant homology to any other protein previously identified, whereas *FLC* encodes a MADS-box transcription factor. FRI positively regulates the expression of *FLC*, whereas the FLC protein negatively regulates the expression of other transcription factors involved in the regulation of flowering such as *SOC1*. Further molecular characterization of *FLC* is showing that it is a central integrator of flowering signals from different pathways (Sheldon et al. 2000). Other loci encoding phytochromes and cryptochromes involved in the flowering response to photoperiod have been also identified (Aukerman et al. 1997; Maloof et al. 2001; Mouradov et al. 2002), leading to a progressive clarification of the processes involved in defining the flowering time and highlighting the importance of natural variation investigation in revealing intricate molecular networks.

9. Inferring biological networks

A key aim of postgenomic research is to systematically catalogue all molecules and their interactions within a living cell. There is a clear need to understand how these molecules and the interactions between them determine the function of the enormously complex cell machinery, both in isolation and when surrounded by other cells forming tissues, organs and whole organisms. Although the study of genetic variation, certainly, contributes to the identification of molecular networks, the large datasets generated by high-throughput technologies, the "omics" technologies, represent a great resource to network biology. Bioformatics tools have been developed to collect and organize the datasets provided by transcriptomics, proteomics and metabolomics analyses (see above), however it is necessary to combine them with a portfolio of interpretation tools able to integrate the multiparameter raw data and link them to biological contexts. Examples of such tools are GENMAPP (Dahlquist et al. 2002), Pathway studio (Nikitin et al. 2003), PATHWAY Processor (Grosu et al. 2002) and BIOMINER (Sirava et al. 2002). However their usefulness for plant datasets is restricted, since they were developed for microbial or animal systems. A first plant-specific application, aimed to integrate transcriptomics data to metabolism, is represented by AraCyc (Mueller et al. 2003). The database (Table 1.1) currently contains about 2000 gene annotations in 117 individual pathways. The pathways are summarised figuratively on an overview map, many are available as detailed diagrams, and a tool, the AraCyc Expression Viewer, allows the user to overlay mRNA expression data on the AraCyc pathway diagrams. One of the comprehensive open-source software packages that allows integration of Arabidopsis transcriptomics and metabolomics data is MAPMAN (Thimm et al. 2004). Within MAPMAN (Table 1.1), Arabidopsis genes are grouped in 200 hierarchical categories by a module called TRANSCRIPTSCAVENGER, and hundreds of metabolites are linked to pathways using the METABOLITESCAVENGER module. The IMAGEANNOTATOR module allows the uploading of experimental data, resulting in a quick overview of the pathways together with transcript and/or metabolite contents. An impressive tool in development is represented by MetNet (Wurtele et al. 2003). This bioinformatics package is able to model metabolic and regulatory networks. Currently, only a limited amount of data have been uploaded into MetNet, but the first full production version promises to be an invaluable tool for Arabidopsis researchers. BioPathAt (Lange and Ghassemian 2005) most probably represents the first bioinformatics tool able to integrate transcriptomics, proteomics, and metabolomics data in the context of well-annotated

biochemical pathways. In particular, Arabidopsis metabolic pathways have been generated based on the apparent coding capacity of the entirely sequenced Arabidopsis genome and assembled in the BioPathAtMAPS module. A gene list for the enzymes involved in the different pathways has been compiled using literature keyword and sequence-based searches in the TAIR database resulting in the BioPathAtDB module. Roughly 1500 genes/enzymes are present in this module and for all of them the subcellular localization was predicted using the PSORT and TargetP programs. Additionally, information on genes/enzymes expression patterns (organ- and tissue-specificity of transcript abundance), enzyme presence (based on proteomics data), enzyme activity (based on biochemical assays with purified, native proteins or crude protein extracts), and biochemical characteristics of recombinantly expressed isozymes (substrate specificity) are added. A complementary database containing information regarding the organ- and tissue-specific pool sizes of metabolites involved in Arabidopsis biochemical pathways (BioPathAtMETDB) is also available, together with gene-protein, and protein-protein interaction data. Dynamic boxes of different colours, placed on the biochemical pathway maps, are used to visualise patterns of RNA abundances, protein expression and metabolite pools. Additionally, enzyme activators and repressors are connected to biochemical pathways by coloured lines, providing insights into the networks that regulate metabolic pathways. Taken together, the computational tools support the researchers with a multidimensional representation of metabolic networks that certainly help to manage the data complexity and to enhance the knowledge on plant biology.

10. Conclusions

Plant genomics research has entered the phase of high-throughput gene function characterization due to the development of essential genetic tools, including comprehensive sets of sequence-indexed mutant collections, and the employment of the "omics" technologies. Global genome data, such as transcriptome atlases, are providing a holistic picture of gene expression regulation. Proteomics analyses are defining spatial and temporal localization of proteins as well as their specific dependence upon environmental conditions. In addition, the progressive development of methods to probe protein-protein and DNA-protein interactions, as well as posttranslational modifications, with high coverage and reliability will contribute to the modeling of cellular dynamics. Certainly, metabolomics investigations are already bridging the gap between gene products and experimental

phenotypes. Throughout recent years it has become increasingly clear, however, that each method has its inherent limitations and none of them alone suffices to unequivocally assign functions to genes. In order to take full benefit from functional genomics, the vast and increasing amount of disparate data types needs to be interconnected and stored in central databases, where information concerning gene sequence, gene expression, protein function, protein interaction, protein localization, phenotype of loss of function line, and metabolic perturbation are linked together. Progress in computational studies of existing data are, therefore, of great importance for data integration as well as for the identification of fundamental properties upon which biological model can be built. The long term goal, as suggested in the ambitious "2010 project" (Chory et al. 2000) is "to understand every molecular interaction in every cell throughout a plant lifecycle. […]. The ultimate expression of our goal is nothing short of a virtual plant which one could observe growing on a computer screen, stopping this process at any point in that development, and with the click of a computer mouse, accessing all the genetic information expressed in any organ or cell under a variety of environmental conditions". Work on other plant species will certainly benefit from Arabidopsis research, both in the use of functional data and in research methodology. Rice, for instance, represents a solid platform for transferring Arabidopsis knowledge and enhancing our understanding of crop species.

References

Abdallah F, Salamini F, Leister D (2000) A prediction of the size and evolutionary origin of the proteome of chloroplasts of *Arabidopsis*. Trends Plant Sci 5: 141-142

Alexandersson E, Saalbach G, Larsson C, kjellbom P (2004) Arabidopsis plasma membrane proteomics identifies components of transport, signal transduction and membrane trafficking. Plant Cell Physiol 45: 1543-1556

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. Science 301: 653-657

Aukerman MJ, Hirschfeld M, Wester L, Weaver M, Clack T, Amasino RM, Sharrock RA (1997) A deletion in the PHYD gene of the Arabidopsis Wassilewskija ecotype defines a role for phytochrome D in red/far-red light sensing. Plant Cell 9: 1317-1326

Bannai H, Tamada Y, Maruyama O, Nakai K, Miyano S (2002) Extensive feature detection of N-terminal protein sorting signals. Bioinformatics 18: 298-305

Bellafiore S, Barneche F, Peltier G, Rochaix JD (2005) State transitions and light adaptation require chloroplast thylakoid protein kinase STN7. Nature 433: 892-895

Bhalla R, Narasimhan K, Swarup S (2005) Metabolomics and its role in understanding cellular responses in plants. Plant Cell Rep 24: 562-571

Bligny R, Douce R (2001) NMR and plant metabolism. Curr Opin Plant Biol 4: 191-196

Borevitz JO, Ecker JR (2004) Plant genomics: The third wave. Annu Rev Genomics Hum Genet 5: 443-477

Borevitz JO, Liang D, Plouffe D, Chang HS, Zhu T et al. (2003) Large-scale identification of single-feature polymorphisms in complex genomes. Genome Res 13: 513-523

Brazma A, Parkinson H, Sarkans U, Shojatalab M, Milo J et al. (2003) ArrayExpress--a public repository for microarray gene expression data at the EBI. Nucleic Acids Res 31: 68-71

Brown PO, Botstein D (1999) Exploring the new world of the genome with DNA microarrays. Nat Genet 21: 33-37

Brown SC, Kruppa G, Dasseux JL (2005) Metabolomics applications of FT-ICR mass spectrometry. Mass Spectrom Rev 24: 223-231

Carter GW (2005) Inferring network interactions within a cell. Brief Bioinform 6: 380-389

Carter C, Pan S, Zouhar J, Avila EL, Girke T, Raikhel NV (2004) The vegetative vacuole proteome of *Arabidopsis thaliana* reveals predicted and unexpected proteins. Plant Cell 16: 3285-303

Chory J, Ecker JR, Briggs S, Caboche M, Coruzzi GM et al. (2000) National Science Foundation-Sponsored Workshop Report: "The 2010 Project" functional genomics and the virtual plant. A blueprint for understanding how plants are built and how to improve them. Plant Physiol 123: 423-426

Cook D, Fowler S, Fiehn O, Thomashow MF (2004) A prominent role for the CBF cold response pathway in configuring the low-temperature metabolome of *Arabidopsis*. Proc Natl Acad Sci U S A 101: 15243-15248

Craigon DJ, James N, Okyere J, Higgins J, Jotham J, May S (2004) NASCArrays: a repository for microarray data generated by NASC's transcriptomics service. Nucleic Acids Res 32: 575-577

Cutler SR, Ehrhardt DW, Griffitts JS, Somerville CR (2000) Random GFP::cDNA fusions enable visualization of subcellular structures in cells of *Arabidopsis* at a high frequency. Proc Natl Acad Sci U S A 97: 3718-3723

Dahlquist KD, Salomonis N, Vranizan K, Lawlor SC, Conklin BR (2002) GenMAPP, a new tool for viewing and analyzing microarray data on biological pathways. Nat Genet 31: 19-20

Edgar R, Domrachev M, Lash AE (2002) Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res 30: 207-210

Emanuelsson O, Nielsen H, von Heijne G (1999) ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. Protein Sci 8: 978-984

Emanuelsson O, Nielsen H, Brunak S, von Heijne G (2000) Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. J Mol Biol 300: 1005-1016

Eubel H, Jansch L, Braun HP (2003) New insights into the respiratory chain of plant mitochondria. Supercomplexes and a unique composition of complex II. Plant Physiol 133: 274-286

Feldmann KA, Malmberg RL, Dean C (1994) Mutagenesis in *Arabidopsis*. In *Arabidopsis* (Meyerowitz E and Somerville CR eds) Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp 137-172

Ferro M, Salvi D, Riviere-Rolland H, Vermat T, Seigneurin-Berny D, Grunwald D, Garin J, Joyard J, Rolland N (2002) Integral membrane proteins of the chloroplast envelope: identification and subcellular localization of new transporters. Proc Natl Acad Sci U S A 99: 11487-11492

Ferro M, Salvi D, Brugiere S, Miras S, Kowalski S, Louwagie M, Garin J, Joyard J, Rolland N (2003) Proteomics of the chloroplast envelope membranes from *Arabidopsis thaliana*. Mol Cell Proteomics 2: 325-345

Fields S, Song O (1989) A novel genetic system to detect protein-protein interactions. Nature 340: 245-246

Fiehn O (2002) Metabolomics – the link between genotypes and phenotypes. Plant Mol Biol 89: 235-249

Frey M, Stettner C, Gierl A (1998) A general method for gene isolation in tagging approaches: amplification of insertion mutagenised sites (AIMS). Plant J 13: 717-721

Fukao Y, Hayashi M, Nishimura M (2002) Proteomic analysis of leaf peroxisomal proteins in greening cotyledons of *Arabidopsis thaliana*. Plant Cell Physiol 43: 689-696

Fukusaki E, Kobayashi A (2005) Plant metabolomics: potential for practical operation. J Biosci Bioeng 100: 347-354

Goff SA, Ricke D, Lan TH, Presting G, Wang R et al. (2002) A draft sequence of the rice genome (Oryza sativa L. ssp. japonica). Science 296: 92-100

Gong M, Rong YS (2003) Targeting multi-cellular organisms. Curr Opin Genet Dev 13:215-220

Greene EA, Codomo CA, Taylor NE, Henikoff JG, Till BJ et al. (2003) Spectrum of chemically induced mutations from a large-scale reverse-genetic screen in *Arabidopsis*. Genetics 164: 731-40

Grosu P, Townsend JP, Hartl DL, Cavalieri D (2002) Pathway Processor: a tool for integrating whole-genome expression results into metabolic networks. Genome Res 12: 1121-1126

Hackbusch J, Richter K, Muller J, Salamini F, Uhrig J (2005) A central role of *Arabidopsis thaliana* ovate family proteins in networking and subcellular localization of 3-aa loop extension homeodomain proteins. Proc Natl Acad Sci U S A 102: 4908-4912

Hannon GJ (2002) RNA interference. Nature 418: 244-251

Heazlewood JL, Tonti-Filippini JS, Gout AM, Day DA, Whelan J, Millar AH (2004) Experimental analysis of the Arabidopsis mitochondrial proteome highlights signaling and regulatory components, provides assessment of targeting prediction programs, and indicates plant-specific mitochondrial proteins. Plant Cell 16: 241-256

Heazlewood JL, Tonti-Filippini J, Verboom RE, Millar AH (2005) Combining experimental and predicted datasets for determination of the subcellular location of proteins in *Arabidopsis*. Plant Physiol 139: 598-609

Herald VL, Heazlewood JL, Day DA, Millar AH (2003) Proteomic identification of divalent metal cation binding proteins in plant mitochondria. FEBS Lett 537: 96-100

Holtfort H, Guitton MC, Reski R (2002) Plant functional genomics. Naturwissenschaften 89: 235-249

Holub EB (2001) The arms race is ancient history in *Arabidopsis*, the wildflower. Nat Rev Genet 2: 516-527

Huber SC, Hardin SC (2004) Numerous posttranslational modifications provide opportunities for the intricate regulation of metabolic enzymes at multiple levels. Curr Opin Plant Biol 7: 318-322

Jensen ON (2000) Modification-specific proteomics: strategies for systematic studies of post-translationally modified proteins. Proteomics: A trend guide. Blackstock W, Mann M (eds) Elsevier Science London

Johanson U, West J, Lister C, Michaels S, Amasino RM, Dean C (2000) Molecular analysis of FRIGIDA, a major determinant of natural variation in Arabidopsis flowering time. Science 290: 344-347

Kant MR, Ament K, Sabelis MW, Haring MA (2004) Differential timing of spider mite-induced direct and indirect defenses in tomato plants. Plant Physiol. 135: 483-495

Kieselbach T, Hagman-Andersson B, Schröder WP (1998) The thylakoid lumen of chloroplasts. Isolation and characterization. J Biol Chem 273: 6710-6716

Koornneef M, Alonso-Blanco C, Vreugdenhil D (2004) Naturally occurring genetic variation in *Arabidopsis thaliana*. Annu Rev Plant Biol 55: 141-172

Kopka J, Schauer N, Krueger S, Birkemeyer C, Usadel B et al. (2005) GMD@CSB.DB: the Golm Metabolome Database. Bioinformatics 21: 1635-1638

Koroleva OA, Tomlinson ML, Leader D, Shaw P, Doonan JH (2005) High-throughput protein localization in *Arabidopsis* using Agrobacterium-mediated transient expression of GFP-ORF fusions. Plant J 41: 162-174

Kruft V, Eubel H, Jansch L, Werhahn W, Braun HP (2001) Proteomic approach to identify novel mitochondrial proteins in *Arabidopsis*. Plant Physiol 127: 1694-1710

Kuromori T, Hirayama T, Kiyosue Y, Takabe H, Mizukado S et al. (2004) A collection of 11800 single-copy Ds transposon insertion lines in *Arabidopsis*. Plant J 37: 897-905

Lange BM, Ghassemian M (2005) Comprehensive post-genomic data analysis approaches integrating biochemical pathway maps. Phytochemistry 66: 413-451

Li Y, Rosso MG, Strizhov N, Viehoever P, Weisshaar B (2003) GABI-Kat SimpleSearch: a flanking sequence tag (FST) database for the identification of T-DNA insertion mutants in *Arabidopsis thaliana*. Bioinformatics 19: 1441-1442

Liu YG, Whittier RF (1995) Thermal asymmetric interlaced PCR: automatable amplification and sequencing of insert end fragments from P1 and YAC clones for chromosome walking. Genomics 25: 674-681

Lockhart DJ, Winzeler EA (2000) Genomics, gene expression and DNA arrays. Nature 405: 827-836

Maloof JN, Borevitz JO, Dabi T, Lutes J, Nehring RB et al. (2001) Natural variation in light sensitivity of *Arabidopsis*. Nat Genet 29: 441-446

Mann M, Jensen ON (2003) Proteomic analysis of post-translational modifications. Nat Biotechnol 21: 255-261

Meinke DW, Meinke LK, Showalter TC, Schissel AM, Mueller LA, Tzafrir I (2003) A sequence-based map of Arabidopsis genes with mutant phenotypes. Plant Physiol 131: 409-418

Meyers BC, Kozik A, Griego A, Kuang H, Michelmore RW (2003) Genome-wide analysis of NBS-LRR-encoding genes in *Arabidopsis*. Plant Cell 15: 809-834

Michaels S, Amasino RM (1999) FLOWERING LOCUS C encodes a novel MADS domain protein that acts as a repressor of flowering. Plant Cell 11: 949-956

Millar AH (2004) Location, location, location: surveying the intracellular real estate with proteomics in plants. Funct Plant Biol 31: 563-571

Millar AH, Sweetlove LJ, Giege P, Leaver CJ (2001) Analysis of the Arabidopsis mitochondrial proteome. Plant Physiol 127: 1711-1727

Mouradov A, Cremer F, Coupland G (2002) Control of flowering time: interacting pathways as a basis for diversity. Plant Cell 14 :111-130

Mueller LA, Zhang P, Rhee SY (2003) AraCyc: a biochemical pathway database for *Arabidopsis*. Plant Physiol 132: 453-460

Nikiforova VJ, Kopka J, Tolstikov V, Fiehn O, Hopkins L, Hawkesford MJ, Hesse H, Hoefgen R (2005) Systems rebalancing of metabolism in response to sulfur deprivation, as revealed by metabolome analysis of Arabidopsis plants. Plant Physiol 138: 304-318

Nikitin A, Egorov S, Daraselia N, Mazo I (2003) Pathway studio - the analysis and navigation of molecular networks Bioinformatics Applications Note 19: 1-3

Nuhse TS, Stensballe A, Jensen ON, Peck SC (2003) Large-scale analysis of in vivo phosphorylated membrane proteins by immobilized metal ion affinity chromatography and mass spectrometry. Mol Cell Proteomics 2: 1234-1243

Nuhse TS, Stensballe A, Jensen ON, Peck SC (2004) Phosphoproteomics of the Arabidopsis plasma membrane and a new phosphorylation site database. Plant Cell 16: 2394-2405

Ogata H, Goto S, Sato K, Fujibuchi W, Bono H, Kanehisa M (1999) KEGG: Kyoto Encyclopedia of Genes and Genomes. Nucleic Acids Res 27: 29-34

Oliver S (2000) Guilt-by-association goes global. Nature 403: 601-603

Ostergaard L, Yanofsky MF (2004) Establishing gene function by mutagenesis in *Arabidopsis thaliana*. Plant J 39: 682-696

Parinov S, Sundaresan V (2000) Functional genomics in Arabidopsis: large-scale insertional mutagenesis complements the genome sequencing project. Curr Opin Biotechnol 11: 157-161

Pendle AF, Clark GP, Boon R, Lewandowska D, Lam YW et al. (2005) Proteomic analysis of the Arabidopsis nucleolus suggests novel nucleolar functions. Mol Biol Cell 16: 260-269

Peters JL, Cnudde F, Gerats T (2003) Forward genetics and map-based cloning approaches. Trends Plant Sci 8: 484-491

Prime TA, Sherrier DJ, Mahon P, Packman LC, Dupree P (2000) A proteomic analysis of organelles from *Arabidopsis thaliana*. Electrophoresis 21: 3488-3499

Raamsdonk LM, Teusink B, Broadhurst D, Zhang N, Hayes A et al. (2001) A functional genomics strategy that uses metabolome data to reveal the phenotype of silent mutations. Nat Biotechnol 19: 45-50

Redman JC, Haas BJ, Tanimoto G, Town CD (2004) Development and evaluation of an Arabidopsis whole genome Affymetrix probe array. Plant J 38: 545-561

Richly E, Leister D (2004) An improved prediction of chloroplast proteins reveals diversities and commonalities in the chloroplast proteomes of *Arabidopsis* and rice. Gene 329: 11-16

Roberts, JK (2002) Proteomics and a future generation of plant molecular biologists. Plant Mol Biol 48: 143-154

Roeder AH, Ferrandiz C, Yanofsky MF (2003) The role of the REPLUMLESS homeodomain protein in patterning the *Arabidopsis* fruit. Curr Biol 13: 1630-1635

Samson F, Brunaud V, Balzergue S, Dubreucq B, Lepiniec L et al. (2002) FLAGdb/FST: a database of mapped flanking insertion sites (FSTs) of *Arabidopsis thaliana* T-DNA transformants. Nucleic Acids Res 30: 94-97

Santoni V, Doumas P, Rouquie D, Mansion M, Rabilloud T, Rossignol M (1999) Large scale characterization of plant plasma membrane proteins. Biochimie 81: 655-661

Schein AI, Kissinger JC, Ungar LH (2001) Chloroplast transit peptide prediction: a peek inside the black box. Nucleic Acids Res 29: E82

Schomburg I, Chang A, Ebeling C, Gremse M, Heldt C, Huhn G, Schomburg D (2004) BRENDA, the enzyme database: updates and major new developments. Nucleic Acids Res 32: 431-433

Schubert M, Petersson UA, Haas BJ, Funk C, Schroder WP, Kieselbach T (2002) Proteome map of the chloroplast lumen of *Arabidopsis thaliana*. J Biol Chem 277: 8354-8365

Schweitzer B, Predki P, Snyder M (2003) Microarrays to characterize protein interactions on a whole-proteome scale. Proteomics 3: 2190-2199

Seigneurin-Berny, D., Rolland, N., Garin, J. and Joyard, J (1999) Technical Advance: Differential extraction of hydrophobic proteins from chloroplast envelope membranes: a subcellular-specific proteomic approach to identify rare intrinsic membrane proteins. Plant J 19: 217-228

Sheldon CC, Rouse DT, Finnegan EJ, Peacock WJ, Dennis ES (2000) The molecular basis of vernalization: the central role of FLOWERING LOCUS C (FLC). Proc Natl Acad Sci U S A 97: 3753-3758

Sirava M, Schafer T, Eiglsperger M, Kaufmann M, Kohlbacher O, Bornberg-Bauer E, Lenhof HP (2002) BioMiner--modeling, analyzing, and visualizing biochemical pathways and networks. Bioinformatics 18: 219-230

Smith MG, Jona G, Ptacek J, Devgan G, Zhu H, Zhu X, Snyder M (2005) Global analysis of protein function using protein microarrays. Mech Ageing Dev 126:171-175

The Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 408: 796-815

Thimm O, Blasing O, Gibon Y, Nagel A, Meyer S, Kruger P, Selbig J, Muller LA, Rhee SY, Stitt M (2004) MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. Plant J 37: 914-939

Zimmermann P, Hirsch-Hoffmann M, Hennig L, Gruissem W (2004) GENEVESTIGATOR. Arabidopsis microarray database and analysis toolbox. Plant Physiol 136: 2621-2632

Zimmermann P, Hennig L, Gruissem W (2005) Gene-expression analysis and network discovery using Genevestigator. Trends Plant Sci 10: 407-409

Werhahn W, Braun HP (2002) Biochemical dissection of the mitochondrial proteome from *Arabidopsis thaliana* by three-dimensional gel electrophoresis. Electrophoresis 23: 640-646

Wouters FS, Verveer PJ, Bastiaens PIH (2001) Imaging biochemistry inside cells. Trends Cell Biol 11: 203-211

Wurtele ES, Li J, Diao L, Zhang H, Foster CM et al. (2003) MetNet: Software to build and modelt he biogenetic lattice of *Arabidopsis.* Comparative and Functional Genomics 4: 239-245

Yu J, Hu S, Wang J, Wong GK, Li S et al. (2002) A draft sequence of the rice genome (*Oryza sativa L. ssp. indica*). Science 296: 79-92

Yu J, Wang J, Lin W, Li S, Li H et al. (2005) The genome of *Oryza sativa*: a history of duplication. PLoS Biol 3: 266-281

2

Improving photosynthesis and yield potential

Jeffrey S. Amthor

U.S. Department of Energy, Germantown, Maryland, USA
(e-mail: jeff.amthor@science.doe.gov)

1. Introduction

Crop yield is fundamentally related to the (a) amount of solar radiation absorbed; (b) efficiency of solar energy use in photosynthesis; (c) translocation of photosynthate to sinks, especially sinks later harvested; (d) capacity for growth in sinks; (e) efficiency of converting photosynthate to new biomass; and (f) metabolic cost of maintenance. *Yield potential* has been defined as the yield of a cultivar grown in an environment to which it is suited, with ample nutrients and water, and with pests, diseases, weeds, lodging, and other stresses effectively controlled (Evans and Fischer 1999). In principle, it integrates the genetic limitations on (a)–(f) as expressed in yield. It is an upper limit to on-farm yield of a cultivar, based on empirical study of that cultivar. As distinct from yield potential, *potential yield* is the yield theoretically possible from a given amount of absorbed solar energy and a specified crop biochemical composition. It is a theoretical construct based on known stoichiometries of biochemical reactions.

Population growth, increased standards of living, and expanding uses of crop products in industrial processes may require significant increases in yield potential of major crops. In recent years, however, yield potential has increased slowly or not at all (Cassman et al. 2003). Genetic engineering offers opportunities to more rapidly increase yield potential — limited ultimately by potential yield — because genes from any organism, not just

P. Ranalli (ed.), Improvement of Crop Plants for Industrial End Uses, 27–58.

from sexually compatible plants, can be spliced into a crop's DNA. Moreover, genes might be improved by site-directed mutagenesis.

An idea underlying the yield potential concept is that there are genes related to yield under favorable conditions and other genes related to stress tolerance or avoidance (Evans and Fischer 1999). While improving yield potential is the focus herein, improving avoidance or tolerance of abiotic and biotic stresses is also critical to overall crop genetic improvement. In this regard, it is important that increased yield potential may enhance yield in stressful environments (Richards 2000).

This chapter suggests tactics, based in part on the possibilities of genetic engineering, for closing the gap between potential yield and yield potential. Photosynthesis is emphasized because a large fraction of the possible gain in yield potential is associated with greater photosynthesis, but improvements to any of items (a)–(f) might enhance yield potential.

2. Absorbing solar radiation

Solar irradiance at earth's surface (I_S, J m^{-2} s^{-1}) is the energy source for photosynthesis, but not all wavelengths of solar radiation are equally useful. Only radiation in the approximately 400–700 nm wave band contributes significantly to crop photosynthesis (Fig. 2.1), so that radiation is called photosynthetically active radiation, or PAR (McCree 1981). Moreover, the rate of photosynthesis is related better to the number of photons (or photon flux) of PAR than to the amount of energy in that PAR. This is because photosynthetic pigments absorb radiation one photon at a time, and each photon excites only one electron in a primary photochemical reaction.

Photosynthetic solar irradiance (I_P, J m^{-2} s^{-1}) is the part of I_S in the PAR wave band. Typically, $I_P = 0.50\ (\pm 0.02)\ I_S$. At a given place and time, I_P (and the associated photon flux of PAR) depends on earth-sun geometry and state of the atmosphere. In the tropics I_P varies little from month to month. Subtropical regions with limited cloud cover receive more solar radiation annually than many tropical locations, and have a modest seasonal cycle. While annual totals are smaller at midlatitudes than they are in the tropics, monthly totals of I_P at midlatitudes in summer can be larger than they are at tropical locations.

Only the fraction of I_P absorbed by a crop ($a_{P,crop}$) can be used for crop photosynthesis, so the product $a_{P,crop}\ I_P$ places an upper limit on the amount of crop photosynthesis possible. The remainder of I_P is reflected up to the sky ($r_{P,sky}\ I_P$) or absorbed by weeds or soil ($a_{P,noncrop}\ I_P$). For a sparse canopy, $a_{P,crop}$ is small, but for a healthy, dense, weed-free crop canopy, it might be about 0.93 (corresponding to $r_{P,sky} \approx 0.06$ and $a_{P,noncrop} \approx 0.01$).

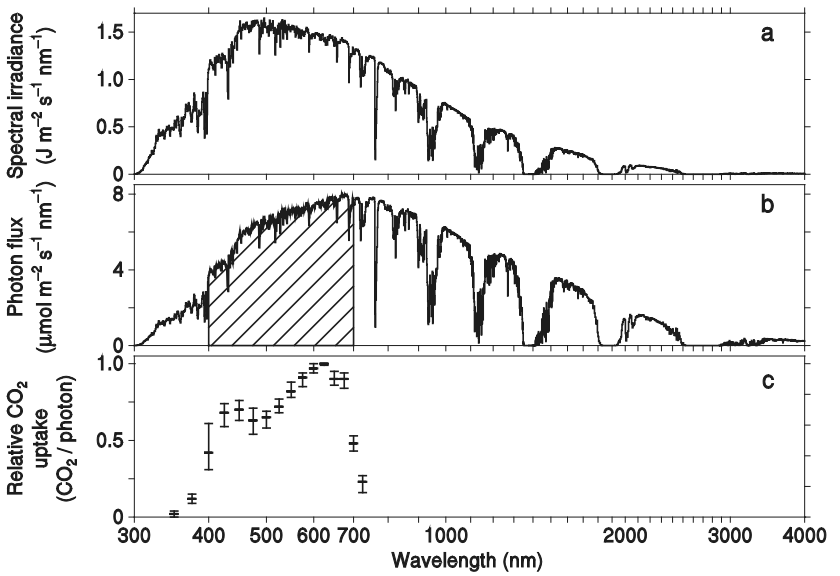


Fig. 2.1. (a) American Society for Testing and Materials G173-03 reference global solar spectral irradiance beneath the 1976 U.S. Standard Atmosphere with air mass = 1.5 (from http://rredc.nrel.gov/solar). This spectrum does not apply to any specific time or place. Dips in the spectrum are caused by atmospheric absorption. **(b)** Spectral photon flux density derived from (a), with the 400–700 nm wave band hatched. There are about 4.6 μmoles of photons per joule in the 400–700 nm wave band [energy in a photon (E, J) is given by $E = hc/\lambda$, where h is the Planck constant (662.6×10^{-36} J s), c is speed of light (299.8 Mm s^{-1}), and λ is wavelength (m)]. **(c)** *Relative* photosynthesis (CO_2 uptake) per photon (in 25-nm intervals) *incident* on leaves of eight field-grown crop species. Bars show minimum, mean, and maximum values among the species (tabulated in McCree 1972)

Some of $a_{P,crop}\ I_P$ is absorbed by entities other than photosynthetic pigments and is thus not available for photosynthesis. That fraction can be called *inactive absorption* as contrasted with *active absorption* by photosynthetic pigments. Inactive absorption might account for 10% of absorbed PAR in green leaves (Seybold 1933); the fraction in whole crops is poorly quantified. Reducing inactive absorption could enhance photosynthesis. A tactic is to quantify inactive absorption in crop leaves, determine if it is beneficial or unavoidable, and if not, reduce that absorption.

Although the 400–700 nm wave band is usually used to define the limits of PAR, photons with wavelength shorter than 400 nm can drive photosynthesis, but the epidermis usually absorbs them before they reach chloroplasts (McCree 1981). If epidermal transmittance of 330–400-nm photons could be increased without detriment to a crop, the PAR wave band for that crop would be increased. The potential increase in photosynthesis might be a few percent (see McCree and Keener 1974).

Whole-season photosynthesis and yield are related to whole-season PAR absorption, which depends on canopy duration (Watson 1958; Shibles and Weber 1965; Warren Wilson 1967; Monteith 1977; Lawlor 1995). Factors that can increase whole-season PAR absorption are rapid emergence and leaf expansion to cover the ground early in a crop cycle (leaf growth rate may be limited by low temperature early in the season) and increased leaf and canopy longevity. Rapid leaf expansion would be at the expense of leaf thickness. This might reduce early-season photosynthesis per unit leaf area, but it would normally be offset by increased photosynthesis per unit ground area. Traits for rapid leaf growth and PAR interception were discussed by Richards (2000).

Breeding for longer leaf duration apparently contributed to greater yield potential in several crops (Evans 1993; Tollenaar and Wu 1999), and durum wheat mutants with delayed leaf senescence out-yielded their parental line in a glasshouse environment[1] (Spano et al. 2003). Effects of delayed leaf senescence on yield may be particularly important during grain and tuber filling periods. There is genetic variation in leaf longevity in crop species, and lengthening canopy duration may be the simplest way to increase whole-season photosynthesis through genetic changes (Richards 2000). A potential tradeoff exists between delayed leaf senescence and crop nitrogen use. Maintaining green leaves with large protein contents to the time of crop maturity implies significant nitrogen in crop residue. This can be thought of as inefficient nitrogen use.

Where cold winters occur (i.e., locations with extended periods of mean 24-hour temperature below about 10°C, corresponding roughly to latitudes greater than 30°), crop growth is limited to summers. A goal for such locations is to grow and maintain a healthy canopy during as much of the summer as possible, which corresponds to the period of greatest I_P. Where all or most of the year is favorable for crop growth (i.e., locations that are warm year-round without significant dry periods, or that are irrigated), most of the annual integral of I_P can be used for crop photosynthesis by (a) using crops such as sugarcane or sugarbeet that can grow up to a full year before harvest, or such as alfalfa, which can be harvested repeatedly, or (b) planting a series of crops on the same land during a year. The latter may depend on early maturity by each crop in the series rather than extended growth duration (Evans and Fischer 1999; Dobermann 2000).

[1] Glasshouse experiments can provide important insight into relationships between crop physiology, genetics, and yield. To relate genetic changes to yield potential, however, field trials that include the highest-yielding cultivars are required.

3. Quantum requirement for photosynthesis

The number of photons of PAR that must be absorbed to assimilate a molecule of CO_2 is the *quantum requirement* for photosynthesis. The number of photons of PAR absorbed by a crop, divided by the quantum requirement, is the maximum amount of photosynthesis possible. The minimum theoretical quantum requirement is therefore a central aspect of the determination of potential yield. It differs between C_3 and C_4 crops[2].

The inverse of the quantum requirement is called quantum yield.

3.1 C_3 photosynthesis

A commonly stated theoretical minimum quantum requirement for C_3 photosynthesis is eight photons per CO_2 molecule. This is based on (a) operation of the Q-cycle in chloroplasts (Berry and Rumberg 1999); (b) transport of four protons through a chloroplastic ATP synthase per ADP phosphorylated (Pänke and Rumberg 1997); (c) use of two NADPH and three ATP in the reductive pentose phosphate cycle (RPPC) per CO_2 assimilated [(a) and (b) together imply that exactly eight photons can reduce two $NADP^+$ and phosphorylate three ADP]; and (d) fructose 6-P as end product. But fructose 6-P is not the end product of photosynthesis. Assimilated carbon is not accumulated or transported in that form. Rather, sucrose, starch, raffinose, and related compounds are the main end products.

To synthesize sucrose from fructose 6-P, 0.083 UTP (taken to be equivalent to 0.083 ATP) are needed per carbon. To produce raffinose and starch, 0.11 and 0.17 ATP/carbon are needed, respectively. Cyclic photophosphorylation might supply that ATP with a stoichiometry of two photons per ATP (i.e., assuming transport of two protons across a thylakoid membrane per photon absorbed by that thylakoid and a four proton requirement for ADP phosphorylation). A theoretical minimum quantum requirement for C_3 photosynthesis is thus 8.17 photons/CO_2 with sucrose as end product. This would represent about 26.5% efficiency of PAR use[3].

---

[2] The C_3 crops include alfalfa, barley, cassava, chickpea, clover, coconut, cotton, cowpea, faba bean, field bean, flax, oat, palm, pea, peanut, pigeonpea, rapeseed (canola), rice, rubber, ryegrass, soybean, sugarbeet, sunflower, wheat, and yam. The C_4 crops include amaranth, maize, millet, sorghum, and sugarcane.

[3] With 4.6 mol photons MJ^{-1} in the PAR wave band (Fig. 2.1), 8.17 mol photons (PAR) contain 1.776 MJ. Sucrose contains 0.470 MJ $(mol\ C)^{-1}$, and $0.470/1.776 \approx 0.265$. The other 1.306 MJ in PAR would be converted to some combination of sensible heat and latent heat.

A key property of the C_3 photosynthesis carboxylating enzyme ribulose-1,5-P_2 carboxylase/oxygenase (rubisco) is that CO_2 and O_2 are competitive substrates. Rubisco's oxygenase activity initiates photorespiration. With a closed cycle of photorespiration (including regeneration of ribulose 1,5-P_2 from 3-phosphoglycerate) each oxygenation catalyzed by rubisco results in the release of 0.5 CO_2, the use of 3.4 ATP, and the oxidation of two NADPH equivalents (i.e., one NADPH and two reduced ferredoxin). The CO_2 release represents a loss of solar energy previously converted into chemical bonds. Eight photons can produce two NADPH and three ATP (as above), but additional energy is needed for the "extra" 0.4 ATP per oxygenation. Using cyclic photophosphorylation, 0.8 mol photons can produce 0.4 mol ATP (as above), so a theoretical minimum quantum requirement by the photorespiratory cycle is 8.8 photons per oxygenation (17.6 photons per CO_2 released).

The relative rates of ribulose 1,5-P_2 oxygenation (v_o) and carboxylation (v_c), with each reaction being catalyzed by rubisco, determines the theoretical quantum requirement for net CO_2 assimilation by C_3 photosynthesis as follows: quantum requirement = $0.17 + (8v_c + 8.8v_o)/(v_c - 0.5v_o)$. This applies to sucrose as the end product. The ratio v_o/v_c, and therefore quantum requirement, is positively related to temperature and negatively related to [CO_2] (Fig. 2.2).

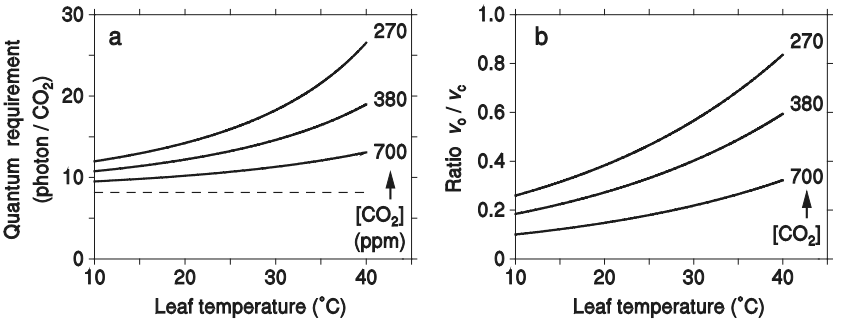


Fig. 2.2. (a) Theoretical quantum requirement for C_3 photosynthesis producing sucrose (—) as a function of temperature for three ambient [CO_2]s with only active absorption of PAR. The lines indicate the balance of rubisco-catalyzed carboxylations and photorespiratory decarboxylations. The 270 ppm [CO_2] corresponds to the preindustrial atmosphere, the 380 ppm [CO_2] corresponds to the contemporary atmosphere, and 700 ppm is a possible atmospheric [CO_2] 90–100 years in the future. A typical ratio of intercellular to ambient [CO_2]s in C_3 leaves was assumed (i.e., 0.7). The dashed line (- - -) is the theoretical minimum quantum requirement without photorespiration (i.e., 8.17 photons/CO_2). **(b)** Theoretical ratio of rubisco oxygenations to carboxylations (v_o/v_c) for conditions as in (a). The fraction of CO_2 assimilated that is subsequently released by photorespiration is $0.5v_o/v_c$.

3.2 C_4 photosynthesis

The C_4 system involves specialized leaf anatomy and adds biochemical reactions (i.e., C_4 cycles) upstream of the RPPC. The system acts to concentrate CO_2 in bundle sheath cells, the location of rubisco in C_4 leaves. This greatly reduces photorespiration in C_4 leaves. Three types of C_4 photosynthesis are usually distinguished, based on the enzyme releasing CO_2 from the C_4 cycle within bundle sheath cells, though the differences between types are not absolute (Leegood 2002). Those enzymes are NADP-malic enzyme (NADP-ME), NAD-ME, and phosphoenolpyruvate carboxykinase (PCK). Maize, sorghum, and sugarcane are NADP-ME-type C_4 crops.

All three types of C_4 photosynthesis involve coordinated activities and interconnections between mesophyll cells (where CO_2 is assimilated in the C_4 cycle) and adjacent bundle sheath cells (where the C_4 cycles release CO_2 and where the RPPC takes place) that encircle leaf veins. The C_4 bundle sheath system is known as Kranz (wreath or ring) anatomy.

The C_4 system requires the production of two NADPH and three ATP per CO_2 assimilated in the RPPC (as for C_3 photosynthesis), and two more ATP per CO_2 moving through the C_4 cycle. The "extra" ATP might come from cyclic photophosphorylation, in the ratio 0.5 ATP/photon (as above), so absorption of four photons could supply the two extra ATP for the C_4 cycle. To synthesize sucrose from CO_2 with the C_4 system would therefore require a minimum of 12.17 photons/CO_2. Some CO_2 leakage from the bundle sheath occurs, however, so the C_4 cycle operates more rapidly (C_4 overcycling) than the RPPC. The amount of C_4 overcycling in C_4 crop species may be of order 0.15 (i.e., 15%) (Hatch et al. 1995), increasing the theoretical quantum requirement of order 0.6 photons/CO_2.

As distinct from C_3 photosynthesis, the quantum requirement for C_4 photosynthesis is largely insensitive to [CO_2] and temperature.

3.3 Comparing C_3 to C_4 and potential to actual

Although C_3 photosynthesis without photorespiration has a smaller quantum requirement than C_4 photosynthesis, once v_o/v_c exceeds about 0.35 in a C_3 leaf, the theoretical quantum requirement for C_4 photosynthesis becomes superior (Table 2.1). This may occur around 25°C (Fig. 2.2).

Observed (i.e., measured or apparent) quantum requirements for CO_2 uptake in unstressed C_3 leaves are larger than theoretical values. For example, sunflower leaf quantum requirement measured in low light (where efficiency is highest) was about 11.5 photons/CO_2 when photorespiration was suppressed by low [O_2] or high [CO_2] (Table 2.2). This is considerably larger than the theoretical value of about 8.2 photons/CO_2. Observed

quantum requirements in C_4 leaves also exceed theoretical values, even after allowing for overcycling of 0.2 (20%) (compare Tables 2.1 and 2.2).

Observed quantum requirements exceed theoretical ones in part because of inactive absorption. With 10% inactive absorption, a theoretical quantum requirement of 8.2 photons/CO_2 (about the minimum for C_3 photosynthesis) increases to 9.1 photons/CO_2. Similarly, a 13.1 photon/CO_2 theoretical value (C_4 photosynthesis with 10% overcycling; Table 2.1) would increase to 14.6 photons/CO_2 with 10% inactive absorption. Also, crop photochemistry is not 100% efficient, and the two photosystems are unlikely to be perfectly coordinated, both of which contribute to differences between potential and actual quantum requirements.

The use of photosynthetically produced NADPH and ATP to support processes in addition to CO_2 assimilation can also affect the measured quantum requirement. An example is daytime NO_3 assimilation in leaves. Effects of light-driven NO_3 assimilation on measured quantum requirement will vary among crops (i.e., depending on how much NO_3 a species assimilates in irradiated leaves), developmental stage, environmental conditions, and the products of NO_3 assimilation (see, e.g., Noctor and Foyer 1998).

Table 2.1. Theoretical minimum quantum requirements for C_3 and maize-type C_4 photosyntheses (synthesis of sucrose with only active absorption of PAR).

System	v_o/v_c of rubisco [oxygenations per carboxylation]	C_4 overcycling [fraction]	Quantum requirement [photons/CO_2]
C_3	0.00[a]	–	8.2
	0.10	–	9.5
	0.20	–	11.0
	0.30	–	12.7
	0.35	–	13.6
	0.40	–	14.6
	0.60	–	19.1
C_4	0.00[a]	0.0[b]	12.2
	0.04[c]	0.0[b]	12.7
	0.04[c]	0.1	13.1
	0.04[c]	0.2	13.5
	0.04[c]	0.3	13.9
	0.04[c]	0.5	14.7

[a]The theoretical lower limit for v_o/v_c is zero (i.e., no photorespiration).
[b]Potential value if CO_2 leakage from bundle sheaths was reduced to zero.
[c]C_4 plants carry out limited photorespiration; the 0.04 value was chosen as representative (e.g., de Veau and Burris 1989; Jenkins et al. 1989).

Table 2.2. Measured leaf-level quantum requirements for C_3 and C_4 photosyntheses (in low light, including any inactive absorption of PAR).

Plant(s)	Quantum requirement [photons/CO_2]	Data source[a]
C_3 *(normal photorespiration)*		
Oat and barley (16°C)	13.7	A
Sunflower (20°C)	13.9–14.3	B
Nine grasses (30°C)	17.9–19.2	A
C_3 *(suppressed photorespiration)*		
Sunflower (20°C)	11.5–11.6	B
Eleven species (28°C)	10.2–11.2	C
C_4		
Eight NADP-ME-type grasses (30°C)	14.5–16.4	A
Three NAD-ME-type grasses (30°C)	16.4–16.7	A
Five PCK-type grasses (30°C)	14.9–16.7	A

[a]A, Ehleringer and Pearcy (1983); B, Sharp et al. (1984); C, Long et al. (1993).

The use of photosynthetic NADPH and ATP to assimilate NO_3 instead of CO_2 during daytime in leaves should not be considered an inefficiency of photosynthesis, but rather an alternative, beneficial use of absorbed PAR. Nonetheless, it is not quantified by CO_2 uptake measurements. Herein, the metabolic cost of NO_3 assimilation is considered part of the substrate requirement for biosynthesis (section 6); i.e., it is considered separately from photosynthesis, even though it may not be so in reality.

Light saturation also increases apparent quantum requirement, often dramatically. For individual C_3 leaves that develop in full sun, photosynthesis can approach light saturation at 15–25% of full sun. Electron transport or carbon metabolism reactions may be near their capacities then. Transport of assimilated carbon out of leaves might also be near its capacity then, which has the potential to cause feedback inhibition of photosynthesis. No matter what causes light saturation of C_3 photosynthesis, it results in a significant increase in the number of photons absorbed per CO_2 molecule assimilated at moderate to high light in isolated C_3 leaves. Light saturation also occurs in C_4 leaves, but the light levels required are higher. This gives a potential advantage to C_4 photosynthesis with high I_P (Fig. 2.3).

Because crop canopies are not usually composed of horizontal leaves, whole-crop photosynthesis is light-saturated only at much higher I_P, and even then the degree of light saturation can be weak (Loomis and Connor 1992).

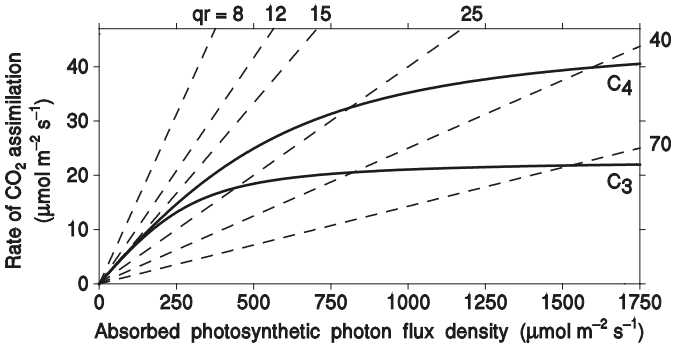


Fig. 2.3. Generalized light response curves on an absorbed photon (PAR) basis for CO_2 assimilation (photosynthesis less photorespiration, but without respiration) by a C_3 and a C_4 leaf. The initial slopes correspond to quantum requirements of 15 photons/CO_2 for both the C_3 and C_4 leaves. The dashed lines indicate CO_2 uptake rates for constant quantum requirements (qr) of 8 (a minimum for C_3 photosynthesis), 12 (a minimum for C_4 photosynthesis), 15, 25, 40, and 70 photons/CO_2. Unshaded horizontal leaves under clear skies can absorb as much as 1750 μmol photons (PAR) m^{-2} (leaf) s^{-1} at midday.

4. Improving photosynthesis

4.1 More rubisco or better rubisco in C_3 crops

If more rubisco — and other photosynthetic enzymes — can be added to C_3 leaves it might allow faster photosynthesis in high light. It might also increase the supply of nitrogen (via mobilization) to growing storage organs during grain or tuber filling periods. This would require greater nitrogen uptake and assimilation, and might require increased nitrogen availability. But even if more nitrogen can be assimilated, there may be limits on how much additional photosynthesis could be obtained in this way. Rubisco contains 20–30% of the nitrogen in C_3 crop leaves, and even more in rice (Evans 1989). It typically accounts for half the *soluble* protein in leaves, and whether much more rubisco could be added to leaves of well-managed crops is unresolved. It was even suggested that there may not be physical capacity to add more rubisco to some leaves (Zhu et al. 2004).

Improving existing rubisco is another possibility for enhancing photosynthesis. Rubisco is an inefficient catalyst of CO_2 assimilation because of its slow turnover number (k_{cat}; a few per second per catalytic site) and low affinity for CO_2. In the present atmosphere, it catalyzes oxygenation of ribulose 1,5-P_2 about 20–60% as rapidly as it catalyzes its carboxylation (Fig. 2.2). This is characterized by the rubisco specificity for CO_2 relative

to O_2, symbolized by τ and equal to $(V_{C,max}K_O)/(V_{O,max}K_C)$. $V_{C,max}$ is the maximum rate of carboxylation, $V_{O,max}$ is the maximum rate of oxygenation, K_O is the Michaelis-Menten constant with respect to dissolved O_2, and K_C is the Michaelis-Menten constant with respect to dissolved CO_2.

An improved rubisco would assimilate more CO_2 per unit mass of rubisco per unit time. Rubiscos from different organisms have (slightly) different amino acid sequences and different properties, including values of τ and k_{cat}. For example, rubiscos from some red algae (Uemura et al. 1997) have much greater τ than found in crops, and C_4 species generally have more efficient rubiscos than C_3 species. Increased τ would reduce quantum requirement and increase C_3 photosynthesis by reducing photorespiration and increasing the rate of carboxylation, even without a change in k_{cat}. A doubling of τ, brought about by increasing $V_{C,max}$ and decreasing $V_{O,max}$ with no change in their sum, might increase wheat leaf photosynthesis 20% at 20°C (Austin 1999). The effect on whole-season photosynthesis could be larger. Faster early-season photosynthesis might increase leaf growth and accelerate canopy closure, thus increasing PAR absorption.

For any given τ, faster k_{cat} could stimulate photosynthesis in high light if cofactors such as P_i were supplied rapidly enough and feedback inhibition of photosynthesis was avoided. The combination of large τ and fast k_{cat} would be most beneficial, but unfortunately, τ and k_{cat} are inversely related across many rubiscos found in nature, including in red algae (Uemura et al. 1997; Spreitzer and Salvucci 2002). Nonetheless, using combinations of τ and k_{cat} observed in nature, simulation modeling indicated that daily crop CO_2 assimilation might be increased 27% simply by replacing the "average C_3 crop rubisco" with the same amount of rubisco from a nongreen-alga, and by 12–17% with rubiscos from C_4 plants (Zhu et al. 2004).

A complication for engineering an improved rubisco is that it is composed of eight large subunits (each of which has a catalytic site) coded for in the chloroplastic genome and eight small subunits coded for in the nuclear genome. Assembling modified subunits in chloroplasts has so far met with difficulty (Parry et al. 2003). The catalytic-site amino acids appear to be conserved and required for maximal activity, implying that engineering an improved rubisco would require non-catalytic-site changes. Also, if a single amino-acid substitution in rubisco could improve photosynthesis, it probably would have been selected already during evolution or created by humans, so it appears that improving rubisco would require multiple changes, perhaps optimized across the whole enzyme complex (Spreitzer and Salvucci 2002). Moreover, the absence of known rubisco mutants with reduced or eliminated oxygenase activity implies that oxygenase activity may be an unavoidable consequence of the reaction mechanism of carboxylation (Leegood 1999). Photorespiration might even benefit a crop during some stresses (see below).

An aggressive tactic for enhancing crop photosynthesis might be to express a high-τ rubisco in lower (shaded) leaves and a high-k_{cat} rubisco in upper (sunlit) leaves, obtained from existing organisms such as algae. A high-τ, fast-k_{cat} rubisco remains the ultimate target for improvement. While improving rubisco will be difficult, the prospects going forward were judged to be "excellent" by Spreitzer and Salvucci (2002), and Parry et al. (2003) wrote that introducing a high-τ rubisco "into crop plants remains a realistic goal". The record on human improvement of rubisco in crops is meager, however, and it might be judged that the probability of significantly increasing yield potential by improving rubisco will be low during the next decade or two.

Improvements might also be made to factors affecting present rubisco. Rubisco activase promotes and maintains the catalytic capacity of rubisco. It reacts with large subunits near their catalytic sites (possibly encircling rubisco as an oligomer), and requires ATP hydrolysis, probably to drive rubisco conformational changes (Portis 2003). The effect of that ATP consumption on the quantum requirement depends on the unknown (perhaps variable) ratio of ATP hydrolyzed to activate rubisco per CO_2 assimilated by rubisco. At high temperature, rubisco activation by activase may not keep pace with rubisco deactivation, possibly limiting photosynthesis above about 30 °C. Increasing activity or stability of rubisco activase at higher temperature might therefore improve the operation of existing rubisco (Crafts-Brandner and Salvucci 2000). Moreover, any engineered change to rubisco may require co-modification of rubisco activase to maintain proper interaction between the two (Spreitzer and Salvucci 2002).

Although the ongoing increase in atmospheric [CO_2] is partly compensating for rubisco's low τ, that [CO_2] increase is slow (atmospheric [CO_2] is now about 380 ppm, increasing about 1.8 ppm/year [Keeling and Whorf 2005]). Modifying rubisco to improve its kinetics should therefore remain a goal. In addition, because [CO_2] inside chloroplasts can be considerably less than [CO_2] in intercellular spaces, increasing the mesophyll conductance (i.e., transfer of CO_2 from intercellular spaces into chloroplasts) could effectively increase CO_2 supply, reduce photorespiration, enhance photosynthesis, and increase yield potential.

4.2 Photoinhibition and photoprotection

Photoinhibition is a reduction of photosynthesis caused by absorption of excess light. It is attributed to thylakoid damage (e.g., damage to the photosystem II reaction center D1 protein) or various "protection" processes (Sage and Reid 1994). Distinguishing effects of actual damage from

effects of protection mechanisms is difficult because both slow photosynthesis. In moderately stressed plants, photoprotection may dominate over thylakoid damage (Demmig-Adams and Adams 1992), but each increases the quantum requirement for photosynthesis.

It is unclear if photoprotection is commonly needed in healthy crops. Young crops with horizontal leaves, or old leaves that become horizontal (Murchie et al. 1999), might be most vulnerable to photodamage. But within developed canopies, leaves are generally not perpendicular to the solar beam, limiting the excess radiation they absorb. Moreover, field crops are "sun" plants, whereas photoinhibition is generally a problem for "shade" leaves. Under stress conditions (e.g., limited soil moisture) photodamage might be common in crops, but the photodamage may be secondary to the stress itself in terms of increased quantum requirement.

If photoprotection is needed and induced in crops, but it persists longer than needed, accelerating the recovery from photoprotection could enhance photosynthesis. The xanthophyll photoprotection system, for example, can be induced by a brief high-light stress but then remain active for hours. More rapid recovery may be desirable, and some higher-yielding rice cultivars may be able to more rapidly recover from artificially induced photoinhibition than traditional cultivars (Wang et al. 2002). It remains unclear, however, whether that faster recovery is related to yield potential. If the recovery rate is important to yield potential, it should be increased, perhaps by genetically modifying the xanthophyll cycle. With respect to engineering changes to C_3-crop rubisco, it is important that photorespiration has the potential to reduce or delay photoinhibition when intercellular $[CO_2]$ is low (e.g., when stomates close because of water deficit). The degree of protection, however, may be modest (Björkman and Demmig-Adams 1994), and because crop mutants with reduced rubisco oxygenase capacity are not known to exist, it is impossible to determine whether rubisco's oxygenase activity per se is beneficial or required by C_3 crops.

Yield gains in some crops apparently have been related in part to increased stomatal conductance, which allows greater evaporative cooling and CO_2 uptake (e.g., Radin et al. 1994; Fischer et al. 1998; Morrison et al. 1999). Greater conductance can reduce the incidence of photodamage in crops (Wang et al. 2005), a trait most relevant to well-watered fields. Greater conductance might also be indicative of greater photosynthesis, perhaps caused by greater sink activity in newer cultivars (see discussion in Richards 2000).

4.3 Leaf angle

Leaf elevation angles that both minimize high incident irradiance on individual leaves and maximize PAR absorption per unit ground area are best. For leaf area index (LAI) less than about two, horizontal leaves may maximize photosynthesis per unit ground area (Loomis et al. 1967). For high LAI (>4), erect leaves can increase photosynthesis by reducing light saturation (and possibly reducing photoinhibition) in the top of the canopy and enhancing photosynthesis lower in the canopy. In principle, this increases the amount of leaf area carrying out efficient (i.e., not light-saturated) photosynthesis and reduces whole-crop quantum requirement. This effect is expected to be largest with high solar elevation, such as during summer or at low latitude (Duncan 1971).

Erect leaves also allow larger LAI by preventing lower leaves from being shaded to the point of senescence, perhaps by keeping daily photosynthesis greater than daily maintenance respiration. This allows greater plant density, which can enhance yield (Loomis et al. 1967; Duvick 2005). The high LAI possible with erect leaves also increases potential for nitrogen storage in leaves per unit ground area. Because grain crops use leaves as a nitrogen source during grain filling, a large LAI can enhance late-season nitrogen supply to growing seeds (Sinclair and Sheehy 1999). Genes for upright leaves are already in the major cereal crops (Reynolds et al. 2000; Richards 2000), but in other crops there is scope for engineering more erect leaves.

5. Maintenance requirement

Living cells require energy for maintenance, and crops obtain that energy from photosynthate. Processes thought to be quantitatively important to the energy cost of crop maintenance include replacement of degraded proteins resulting from their spontaneous breakdown; active transport of metabolites across membranes to counteract "leaks" (i.e., gradient maintenance); active processes involved in acclimation to environmental changes, such as the replacement of one complement of enzymes with another that is better suited to a new environment; and repair of cellular damage by, for example, oxidants in the environment or produced by the plant.

Turnover of biosynthetic enzymes and RNA associated with growth is called "tool maintenance". This is distinguished from other maintenance processes, which are collectively called "structure maintenance" (Penning de Vries et al. 1974). "Maintenance" in this section refers to structure maintenance; tool maintenance is part of the substrate requirement for biosynthesis (next section). "Maintenance respiration" is the CO_2 (and heat)

released by maintenance processes and by respiratory processes supplying ATP, NAD(P)H, or carbon skeleton intermediates to the maintenance processes (Penning de Vries 1975).

Rates of maintenance processes are dynamic, affected by both developmental changes in the crop and changes in the environment. Maintenance respiration rate is probably most strongly related to the amount of protoplasm and to temperature. Maintenance processes are slow in cell walls because those structures do not undergo turnover. Maintenance processes are also probably slow in seeds and tubers because macromolecular turnover and gradient losses are limited there (i.e., the products of biosynthesis in those organs are more stable than is vegetative-cell protoplasm). Shaded leaves too have generally slow rates of maintenance respiration, at least in comparison to leaves in high-light environments. One implication of increasing protein amount in leaves is an increase in maintenance expenditures for protein turnover, although the greater photosynthesis could outweigh the greater respiration. Maintenance may be faster in warm environments than cool ones, but some degree of acclimation of maintenance processes to prevailing temperature is to be expected. Hence, acclimation and adaptation will presumably limit (or eliminate) effects of gradual global warming (associated with increasing atmospheric [CO_2]) on crop maintenance processes.

Experimental data and theoretical calculations indicate that maintenance of vegetative tissue, at moderate temperature, consumes photosynthate at a rate equivalent to at least 1% of the energy content of existing biomass each day (Penning de Vries 1975; Amthor 2000). Estimates are imprecise, but over a crop cycle, maintenance respiration may about equal growth respiration, so an engineered reduction in maintenance respiration might increase growth (Amthor 1989; Loomis and Amthor 1999).

Across different selections of perennial ryegrass, mature-leaf respiration rate (one empirical measure of maintenance respiration rate) was negatively correlated with forage production (Wilson 1975, 1982). Causes of the correlation remain unclear, and the correlation can disappear when the plants are grown at low density (Kraus 1992). A negative correlation between mature-leaf respiration rate and biomass production was also observed in different maize hybrids, but the cause of that correlation and whether differences in maintenance contributed to growth differences is also unclear (Earl and Tollenaar 1998). These results hint that there may be variation in maintenance respiration that can affect growth, but this is still unproven. In any case, an ideal crop would carry out only essential maintenance and it would do so with efficient metabolism. It would minimize spontaneous breakdown of macromolecules and metabolite gradient losses.

It is thus important to establish if (a) all existing maintenance processes are necessary in well-managed crops, (b) existing maintenance processes are efficient, and (c) respiration provides maintenance energy efficiently. A minimum energetic cost of turnover of a unit of protein can in principle be calculated (Amthor 2000), but without information on the minimum amount of turnover required in high-yielding crops, it is impossible to estimate the corresponding minimum energy requirements for that minimum required protein turnover. The same is true for metabolite gradient maintenance and acclimation to environmental variation. In the absence of sufficient understanding of the optimal rates and maximum potential efficiencies of required maintenance processes, a theoretical minimum *maintenance requirement* cannot be calculated. Nonetheless, maintenance is essential, and a speculative value of 15% of photosynthesis is adopted herein as the substrate requirement for minimum whole-plant maintenance needs. In consideration of the lower leaf-protein concentration in C_4 crops, the speculative minimum for C_4 crops is 12% of photosynthesis.

One consideration is that rapid rates of maintenance processes may be important to survival and reproductive success in complex and dynamic environments. For example, frequent and rapid physiological acclimation to both environmental fluctuations and the dynamics of interspecies competition may be critical in natural environments. That frequent and rapid acclimation might depend on rapid background maintenance processes. In well-managed monocultures, however, extensive and frequent acclimation processes may be unnecessary. It is unclear whether past crop selection and breeding reduced the rate, or increased the efficiency, of maintenance processes to their optima for a crop's environment. If they have not, genetic improvement is possible, but the potential for any such improvement will remain unknown until more information about optimal and actual maintenance processes in crops is obtained.

6. Substrate requirement for biosynthesis

The *substrate requirement* for biosynthesis is the amount of photosynthate needed to grow a unit of new biomass. This means that an upper limit on the amount of growth possible is the time-integrated amount of photosynthesis, minus the substrate requirement for maintenance, with the result divided by the substrate requirement for biosynthesis.

Biosynthesis includes catabolic and anabolic phases. The catabolic phase breaks down photosynthate to produce carbon-skeleton intermediates, reduce NAD^+ and $NADP^+$, and phosphorylate ADP. The anabolic phase combines carbon-skeleton intermediates into the macromolecules of

biomass, using the NAD(P)H and ATP provided by the catabolic phase. In addition to photosynthate, soil minerals are needed for biosynthesis, especially nitrogen, which must be reduced for incorporation into biomass. The CO_2 released during biosynthesis of new biomass is called "growth respiration". Most of that CO_2 release is associated with the catabolic phase of biosynthesis, but some anabolic pathways also release CO_2.

Because rapid growth requires rapid growth respiration, slower intrinsic respiration could reduce yield potential. The target for yield-potential improvement is reduced substrate requirement in combination with rapid biosynthesis (and therefore rapid growth respiration).

The theoretical minimum substrate requirement for biosynthesis is calculated by tracing the most efficient pathways from photosynthate to the components of biomass (Penning de Vries et al. 1974, 1983). The major components are structural and storage carbohydrates, proteins, lignins, lipids, and organic acids. The pathways are summed in proportion to biomass composition. Inputs of NAD(P)H and ATP to the pathways, and the amount of substrate catabolized to produce that NAD(P)H and ATP are included. The metabolic cost of transporting carbohydrates, minerals, and other substrates from source organs to sites of biosynthesis is also included, as is the cost of assimilating inorganic nitrogen. The chemical forms of photosynthate and nitrogen used, and composition of the biomass grown, are inputs to such calculations.

Efficiency of biosynthesis is the inverse of substrate requirement. It can be expressed as either biomass (dry) formed per unit mass of substrate used, or as energy contained in the biomass formed per unit energy in the substrate used. The mass/mass ratio is most often used in crop science, but the energy/energy ratio is more relevant to assessing the efficiency of converting solar energy into chemical bonds in new biomass.

Theoretical efficiencies of converting photosynthate in sources into new biomass in storage organs vary from 0.40 to 0.85 kg kg^{-1} across a range of crops (see Fig. 2.4 for assumptions). The theoretical efficiency of energy use in growing those same storage organs is larger and less variable, i.e., covering the range 0.65–0.88 J J^{-1} (Fig. 2.4). Differences between crops arise from differences in their composition, including differences in protein concentration, which defines a *nitrogen requirement* for biosynthesis. For the organs in Fig. 2.4, the nitrogen concentration varied from about 0.5% (cassava) to over 6% (soybean), a more than 12-fold difference in amount of nitrogen that must be assimilated per unit mass of storage organ grown. Calculated energy contents ranged from 16.4 (sugarbeet) to 27.1 (peanut) kJ g^{-1}. The high lipid concentrations in coconut, oil palm, cotton, sunflower, peanut, and soybean contributed to relatively low mass-based biosynthetic efficiencies. The range of compositions of the storage organs in Fig. 2.4 spans many possibilities, including values for most vegetative

organs. Nonetheless, for new crops producing large amounts of novel compounds, it is possible that theoretical substrate requirements (or potential biosynthetic efficiencies) would be outside the range considered here.

Efficiency of substrate use for biosynthesis could be increased only to the extent that it is now less than its potential. Research in the 1970s and 1980s indicated that biosynthesis in crops may be near the theoretical maximum efficiency allowed with known biochemical pathways (Penning de Vries et al. 1983). That research included uncertainty, however, and the actual efficiency of biosynthesis in crops in the field is imprecisely known.

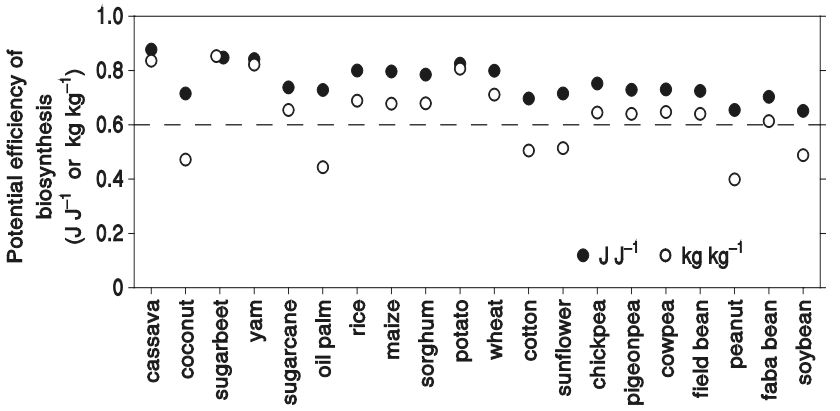


Fig. 2.4. Potential (theoretical) efficiency of biosynthesis (i.e., retention of energy or mass) of crop *storage organs* from sucrose. The organs are tubers and beets (cassava, sugarbeet, yam, potato), sugarcane shoot, fruits (coconut, oil palm), inflorescences with seeds (rice, sorghum, wheat, sunflower), maize cob (70% seed), cotton bole (35% lint), and pods with seeds (chickpea, pigeonpea, cowpea, field bean, peanut, faba bean, soybean). Concentrations of carbohydrates, proteins, lipids, lignins, organic acids, and minerals in the organs are based on Penning de Vries et al. (1983). Organs are arranged from left to right according to increasing protein concentration. Substrate requirements for biosynthesis of each class of biochemical are from Penning de Vries et al. (1983) and Amthor (2003) (for lignins), with small modifications herein for carbohydrates. Tool maintenance estimates are included, and 5% of the sucrose substrate is respired to provide energy to transport the remaining 95% from sources to growing cells in the storage organs. Minerals are supplied as needed, accounting for uptake cost (i.e., sucrose catabolism) in roots. Nitrogen is provided as NO_3 and estimated cost of NO_3 assimilation is included; if some NO_3 assimilation occurs as part of "photosynthesis", biosynthetic efficiency would increase, but the quantum requirement for photosynthesis would increase too. Energy contents of biochemical fractions are based on Appendix. Different assumptions about organ chemical composition or pathways of biosynthesis would result in (generally small) differences in potential efficiencies. The dashed line (– – –) is for visual reference only.

Recent theoretical work revealed potential for variation in the efficiency of biosynthesis of lignins with extant enzymes and pathways (Amthor 2003), implying the possibility for genetic improvement, and the same could be true for other complex biomolecules. To determine if (and how much) improvement is possible for a specific product in a specific crop will require precise measurements of biosynthesis, substrate consumption, and the biosynthetic and respiratory pathways used. If efficiency is not maximal, it could become a target for improvement.

7. Efficiency of PAR use by productive C_3 and C_4 crops: potential and actual

Based on estimates of substrate requirement for biosynthesis and maintenance thought to be representative, the so-called radiation-use efficiency (RUE; grams dry whole-plant biomass grown per joule PAR absorbed) was modeled for various quantum requirements for photosynthesis by wheat at midseason (Loomis and Amthor 1996). Results indicated that potential RUEs would range from 2.3 to 8.6 g MJ^{-1} for quantum requirements ranging from 30 down to 10 photons/CO_2, respectively.[4]

A summary of field measurements of wheat and barley indicated a mean actual RUE of about 3.0 g MJ^{-1}, or 5.1% of energy in absorbed PAR for crops containing 17 kJ g^{-1} (Fischer 1983). A quantum requirement of 25 photons/CO_2 could explain that RUE (Loomis and Amthor 1996). Data on the "high side" of Fischer's (1983) summary indicated that the best crops were about 6.5% efficient at converting absorbed PAR into chemical bonds in new biomass, corresponding to a quantum requirement of 20 photons/CO_2 in the model crop (Loomis and Amthor 1996).

A similar model analysis for maize resulted in potential RUEs ranging from 4.2 to 6.2 g MJ^{-1} for quantum requirements ranging from 18 down to 14 photons/CO_2, respectively (Loomis and Amthor 1999). The more limited range of quantum requirement was used because C_4 quantum requirement is relatively insensitive to the environment and 14 photons/CO_2 may represent a minimum achievable for C_4-crop photosynthesis.

A 5-year field study indicated a mean RUE of about 4.2 g (assumed here to be 10% roots) MJ^{-1} in well-managed maize (Lindquist et al. 2005). That corresponds to the 18-photon-photosynthesis model crop. For model biomass containing 17.6 kJ g^{-1} (Loomis and Amthor 1999), such a crop would be 7.4% efficient at producing chemical bonds in new biomass from absorbed PAR. Data on the "high side" of Lindquist's et al. (2005) summary

[4] Loomis and Amthor (1996, 1999) used 4.4 mol photons MJ^{-1} (PAR) in their analyses. Results herein were recalculated using 4.6 mol MJ^{-1} (Fig. 2.1).

indicated RUEs of about 5.2 g MJ^{-1} (9.1–9.2% efficiency of converting absorbed PAR into biomass energy) during both vegetative and reproductive growth. An RUE of 5.2 g MJ^{-1} would correspond to a quantum requirement of 15.2 photons/CO_2 in the model crop. That quantum requirement is within the range measured for individual C_4 leaves in low light (Table 2.2; in the model crop, the cost of NO_3 assimilation is part of the cost of growth, but in the measured leaves it might be part of photosynthesis).

If the efficiency of biosynthesis is similar in C_3 and C_4 crops, but the observed quantum requirement for photosynthesis is lower in C_4 crops, then C_4 crops should be more productive. (Also, maintenance requirement may be less in C_4 crops because of a smaller protein concentration in leaves.) A summary of standing biomass (not yield) at harvest for highly productive crops supports this supposition [excluding published values likely to be erroneous; see Loomis and Gerakis (1975) and Monteith (1978)], with a qualification about latitude (Fig. 2.5). At latitudes greater than 50° (absolute value), C_3 crops have an apparent advantage, probably because C_4 crops are more sensitive to low temperature than most C_3 crops.

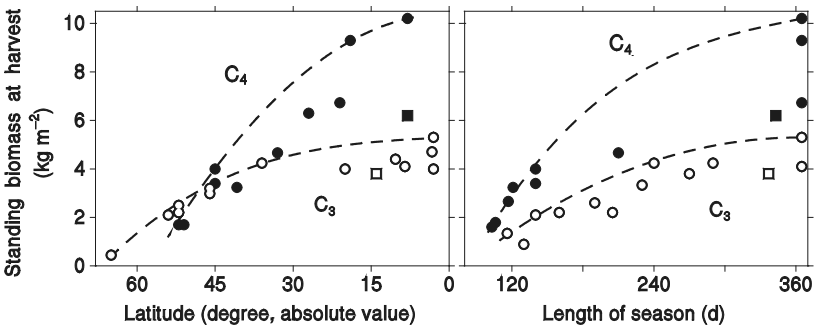


Fig. 2.5. Estimated standing whole-plant biomass at harvest for high-productivity C_3 (○) and C_4 (●) crops as a function of latitude (absolute value) and season length (not all crops shown at left are shown at right and vice versa). Data sources are given in Cooper (1975), Loomis and Gerakis (1975), Loomis (1983), and Smith and Banta (1983). Also shown is the "Comet" soybean crop of Morrison et al. (1999), the 2001 maize crop of Lindquist et al. (2005), a good rice-rice-rice triple-crop at the International Rice Research Institute (□; KG Cassman pers comm), and a good rice-maize-maize triple-crop in East Java (■; A Dobermann pers comm). Total production exceeds values shown because biomass shed prior to harvest is excluded. Lines are drawn by eye to summarize the best C_3 or C_4 crops. Lines in the right panel are concave down in part because average I_P is greater during summer at midlatitudes (about 120-d season) than during the whole year in the tropics

As indicated by Fig. 2.5, the best C_4 crops have faster long-term (i.e., seasonal-average) growth rates than the best C_3 crops. They also have faster short-term, peak growth rates (Loomis and Gerakis 1975; Monteith 1978), at least in warm environments.

The largest standing biomass production estimate in Fig. 2.5 is 10.2 kg m^{-2}, for sugarcane growing all year (Irvine 1983)[5]. A sunny tropical site can receive 3500 MJ (PAR) m^{-2} annually, and a 10 kg m^{-2} sugarcane crop might contain 170–180 MJ m^{-2}, implying about 5.0% efficiency of I_P use averaged over the year. This efficiency is unremarkable compared to the shorter-term efficiencies from Fischer (1983) and Lindquist et al. (2005) above, although it is on an incident rather than absorbed PAR basis.

Based on analysis above, there is large theoretical scope for improving the efficiency of converting absorbed PAR into chemical bonds in C_3 crops. A canopy-scale quantum requirement for C_3 photosynthesis as small as about 11 photons/CO_2 can be contemplated based on reduced photorespiration. This would allow nearly 20% efficiency of converting energy in absorbed PAR into energy in sucrose [i.e., 0.470 MJ (mol C)$^{-1}$/(11 mol photons (mol C)$^{-1}$ / 4.6 mol photons MJ^{-1})≈0.197 J {in sucrose} J^{-1} {in absorbed PAR}]. If the maintenance requirement consumes 15% of the energy in the sucrose product of photosynthesis, and 65–88% of the energy in the remaining sucrose can be retained in new biomass, then an upper limit on converting energy in absorbed PAR into chemical bonds in biomass is 11–15%, depending on biomass composition. For whole wheat plants, the maximum theoretical efficiency of biosynthesis might be about 0.79 J J^{-1}, giving a potential efficiency of converting absorbed PAR into chemical bonds in new biomass of about 13%. This is twice the 6.5% efficiency derived above for the best crops in Fischer's (1983) analysis.

The theoretical scope for improving the efficiency of solar energy use in C_4 crops is smaller because (a) the minimum canopy-scale quantum requirement of C_4 photosynthesis is larger (probably about 14 photons/CO_2) and (b) C_4 crops are already more productive than C_3 crops. Based on composition of whole maize plants, the maximum theoretical energy-based efficiency of biosynthesis may be about 0.82 J J^{-1}. Allowing for a 14-photon/CO_2 minimum canopy-scale quantum requirement and a minimum maintenance requirement of 12% of photosynthesis, the overall potential efficiency of converting energy in absorbed PAR into chemical bonds in new biomass would be about 11%. The measurements by Lindquist et al. (2005) indicate that well-managed maize may now be achieving more than 80% of this potential. To significantly increase biomass accumulation by

[5] Irvine (1983) estimated maximum annual sugarcane biomass production in two other experimental studies at 11.3 and 11.6 kg m^{-2}.

such crops, it may be necessary to increase the amount of PAR absorbed, either through earlier canopy closure or longer canopy duration.

It is noted that potential efficiencies above ignore unavoidable losses, if any, in processes such as leaf senescence, exudation from roots, and leaching from leaves. Also, the maximum measured efficiencies apply to shorter-term periods; they do not, for example, apply to whole years.

8. Engineering C_3 crops to use C_4 photosynthesis

Because C_4 crops are often more productive than C_3 crops at present CO_2 levels, it may be desirable to engineer C_3 crops to use C_4 photosynthesis. It is important, however, that increasing atmospheric [CO_2] is reducing some of the C_4 advantage, although that advantage is unlikely to disappear for many decades. It is also important that the maximum potential efficiency of solar energy use by C_3 crops exceeds the potential by C_4 crops, but if significant improvement to C_3-crop rubisco remains unrealized, C_4 photosynthesis may be preferred. This might not only increase photosynthesis, but could improve water-use efficiency. It could also reduce the nitrogen requirement for photosynthesis, though this could involve a drawback. Using rice as an example, Sheehy (2000) noted that the nitrogen content of C_4 canopies can be insufficient to supply enough nitrogen for high grain yield, at least if root uptake of nitrogen is limited during grain filling.

A number of approaches might be used to engineer C_4 photosynthesis into C_3 crops. They include (see Leegood 2002) introducing into C_3 crops (a) the complete complement of metabolic reactions and structural characteristics of C_4 crops; (b) some form of a single-celled C_4 system; (c) an intracellular CO_2-concentrating compartment, modeled after those found in some algae and cyanobacteria; or (d) a relocation of photorespiratory decarboxylation to the bundle sheath, modeled after that found in C_3–C_4 intermediate species. Expression of genes related to C_4 photosynthesis in C_3 plants can stimulate photosynthesis under some circumstances, but this has so far been unrelated to C_4 photosynthesis per se (e.g., Ku et al. 2001). It is impossible to reliably estimate the length of time required to engineer C_3 crops to use C_4 photosynthesis, but it would probably be decades.

In many cases it would be more expedient to modify existing C_4 crops to produce new products than to convert C_3 crops into C_4 crops. Indeed, the C_4 crop maize has advantages as a production system for industrial products (Stoger et al. 2005) and maize is already a major industrial crop. It has hundreds of nonfood/nonfeed product uses, though they are mostly related to grain starch. Production of different types of products, in desired quantities

and purities, might require considerable research, but advances in genetic engineering would presumably assist in some such efforts.

Improving the low-temperature performance of C_4 crops might allow expansion of their latitudinal range (global warming may contribute modestly to future C_4-crop range expansion too). The NADP-ME-type C_4 plant *Miscanthus* × *giganteus* is relatively tolerant of low temperature and it might serve as a genetic resource for improving the low-temperature tolerance of C_4 crops (Naidu and Long 2004). The time required to make such an improvement in C_4 crops would perhaps be decades.

9. Harvest index and sink activity

A comprehensive definition of harvest index (HI) is the fraction of whole-plant biomass that accumulates in a harvested organ, or the fraction of the whole plant made up of desired biochemicals (see also Irvine 1983). For grain crops, HI generally means the fraction of total aboveground biomass that is contained in grain at the time of harvest. The relationship between HI, biomass accumulation, and yield is simple: HI × biomass = yield.

While increased HI was often the most important factor in past increases in yield potential, how it occurred is incompletely understood. One critical change to grain crops was reduced stem growth, but exactly how that caused greater yield potential is unclear. Perhaps several mechanisms were involved (Evans 1993). The potential (maximum possible) HI has been speculated about (Austin 1999), but it lacks rigorous determination. Nonetheless, for crops already intensively bred for large HI, the scope for further improvement may be small. In those crops, further yield potential increases must come mainly from greater total biomass production. This would require more photosynthesis or more efficient biosynthesis and respiration. In other crops, there may be significant opportunity to increase yield potential by increasing HI. This should be pursued because history shows that such increases in yield potential can be dramatic.

The total amount of substrate imported by harvested sinks places an upper limit on yield. Substrate supply depends on the rate of assimilation in sources (and/or mobilization in intermediate storage pools) and the rate of translocation into sinks. Either could be a limiting process, but phloem *capacity* does not appear to limit transport (Evans 1993). The developmental stage is important, at least in grain crops. Increased photosynthesis prior to flowering often enhances spike growth and number of seeds set, and seed number is often positively related to yield. Increasing the duration of juvenile spike growth might increase seed number, and this might be brought about through genetic modification of photoperiod perception (Reynolds et al. 2000).

The potential importance of this is highlighted by the fact that past increases in yield potential of grain crops was related strongly to more seeds, rather than larger seeds (Evans 1993; Egli 1998; Reynolds et al. 2000; Richards 2000).

It is well established that slow sink activity can feed back to slow photosynthesis (Evans 1993), but with respect to increasing yield potential it is more important to know if increased sink activity (i.e., growth) can stimulate photosynthesis per unit of absorbed PAR. This is difficult to determine, and evidence indicating both negative and affirmative views can be cited (e.g., Evans 1993; Reynolds et al. 2000; Richards 2000; Egli and Bruening 2003). The issue will not be resolved here, and it may depend on species and other variables. In terms of potential yield, photosynthesis is limited by absorption of PAR and photosynthetic pathways and yield is then limited by the (a) fraction of photosynthate that can be transported into desired sinks and (b) amount of growth possible from that transported photosynthate. In principle, sink capacity might affect yield potential, and increasing the capacity for sink growth may be an appropriate target for crop genetic improvement (Reynolds et al. 2000; Richards 2000). A research goal is to quantify, and then experimentally increase, growth potential of desired sinks to determine if such an increase affects yield potential. Sink strength and growth potential may be related in part to phloem unloading, so possible candidates to increase sink activity are overexpression of invertase and sucrose transporters in sinks.

Whether enhanced sink capacity would increase yield potential or not, source activity now typically limits yield. Increasing photosynthesis by increasing PAR absorption or [CO_2] usually enhances yield if soil nutrients are adequate. And even high-yielding grain crops have unfilled seeds, although this may be related to ear structure, a sink attribute possibly amenable to improvement (Sharma-Natu and Ghildiyal 2005). Such observations indicate that source activity limits yield, yet other considerations indicate that there is unused source capacity in crops (see Richards 2000). Again, timing is important. Photosynthesis may be limiting during one period (e.g., prior to flowering), but sink capacity could be limiting during another period. This is particularly difficult to study quantitatively.

Experiments with transgenic plants indicate that changes in sink biochemistry can affect yield. For example, downregulation of plastidial adenylate kinase increased potato tuber growth in a field trial (Regierer et al. 2002), and expression of ADP-glucose pyrophosphorylase with reduced sensitivity to inhibition by P_i increased seed yield in greenhouse-grown rice and wheat (Smidansky et al. 2002, 2003). It is unknown whether such genomic changes could increase yield potential.

For any average rate of desired-product biosynthesis, yield is proportional to duration of that biosynthesis. Duration of both photosynthate transport to harvested sinks and growth in those sinks may therefore limit yield. In many crops the duration of sink growth is controlled by "thermal time" such that warmth reduces growth duration. Lengthening the duration of sink growth by relaxing the relationship between temperature and development, especially in warm climates, might increase yield potential. If duration of sink activity is maximized by extending it to the end of the possible growing period, yield will be limited by substrate supply, efficiency of biosynthesis, or average rate of biosynthesis. (Conversely, rapid maturity may be required when several crops are grown in series in a year.)

Much of the nitrogen (and some of the carbohydrate) used for grain growth in many crops comes from breakdown of older vegetative tissues, especially leaves. Rubisco in particular can be an important source of grain nitrogen. Proteins synthesized in grain from the breakdown products of proteins in vegetative organs are in essence synthesized twice; once in the vegetative organ and again in the grain. The cost of the second synthesis may be balanced by benefits of multiple uses of the same nitrogen, such as in photosynthetic enzymes early in a crop cycle followed by grain protein storage later. This reduces the total amount of nitrogen that must be assimilated. It may also be a limitation on yield — perhaps a vestige of evolution in competitive environments with limited nutrients. Increasing the capacity to acquire and assimilate nitrogen during grain filling might increase yield potential (Austin et al. 1977; Tollenaar and Wu 1999). This might require modified root mass, depth, longevity, or functioning.

10. Final comments

To obtain high yields, crops must assimilate environmental resources (solar radiation, CO_2, and nitrogen are key) at fast rates for significant duration. For this to occur, effective root and canopy systems (including stem structure for leaf display) must be grown, maintained, and protected from pests and stressors. Soil water must be adequate to allow high stomatal conductance and transport of CO_2 into leaves, especially in C_3 crops. Mineral nutrients (especially nitrogen) must be available and assimilated to allow large capacities for photosynthesis and growth.

Modest gains in yield potential might be achieved by reducing inactive absorption of PAR, causing more erect leaf display following canopy closure, or increasing the capacity for stomatal conductance or mesophyll conductance. Even larger yield potential gains might be possible by accelerating early season leaf expansion, increasing leaf or canopy longevity, increasing uptake and assimilation of nitrogen during grain or tuber filling,

reducing photorespiration in C_3 crops, engineering C_3 crops to use C_4 photosynthesis, or increasing HI in crops not previously bred for large HI. More research is needed to better understand other possibilities for improving yield potential. For example, research to quantify actual efficiency of biosynthesis, actual rates and efficiencies of maintenance processes, the minimum maintenance requirements of well-managed crops, and the necessity and extent of photoprotection processes is needed. If any parts of those processes are found to be unnecessary or inefficient, those parts should become targets for elimination or improvement. Although many of the above crop processes and characteristics have been improved in some crops, there remains scope for additional enhancement of yield potential in most cases. An overriding consideration is that multiple factors may co-limit yield potential and the relative importance of those factors can change during the course of a crop cycle and can differ between crop species and environments. So although "no one process provides the master key to greater yield potential" (Evans 1993 p. 169), by understanding factors determining potential yield, the gap between it and yield potential might be closed more rapidly and more efficiently.

By supplementing crop breeding with genetic engineering, not only might the yield potential of present crops be improved more rapidly, but entirely new crops and crop products can be considered. That is, in addition to starch, sugars, rubber, cellulose, lipids, and other traditional industrial-crop products, new products as diverse as biodegradable plastics (e.g., Poirier 1999; Scheller and Conrad 2005) and vaccines (e.g., Santos and Wigdorovitz 2005) can be grown by crops. The possibilities are legion, and the details will vary with each product-crop combination. Because grain crops have advantages as production systems for many products (e.g., Stoger et al. 2005) they will remain valuable for both present and future industrial uses.

Appendix: crop energy content

Plant dry mass may typically contain about 17.8 kJ g^{-1} (Whittaker 1975 p.193). Crop vegetative organs may average 17.0–17.5 kJ g^{-1} (Loomis and Connor 1992 p. 15). Those are heats of combustion (ΔH_C), measured in bomb calorimeters, relative to CO_2 (gas) and H_2O (liquid). Representative ΔH_C values for different classes of biochemicals include (e.g., Loomis and Connor 1992): organic acids, 4–11; carbohydrates, 15.6–17.5 (glucose = 15.6, sucrose = 16.5, starch = cellulose = 17.5); proteins, 22–25; lignins, 26–30; and lipids, 35–40 kJ g^{-1}. Because different compounds have different ΔH_C values, differences in crop composition can cause differences in whole-organ and whole-crop ΔH_Cs. For example, measured ΔH_C values were

16.8, 17.3, 17.6, 18.2, 21.1, and 26.9 kJ g^{-1} for potato tubers, wheat ears, rice ears, maize seeds, soybean pods, and sunflower seeds, respectively (Shinano et al. 1993).

Acknowledgments

Ken Cassman, David Connor, Achim Dobermann, Dennis Egli, M.G. Goodbody, Paul Hanson, David Lawlor, John Lindquist, Bob Loomis, Seth Pritchard, and John Sheehy are thanked for their gracious help.

References

Amthor JS (1989) Respiration and crop productivity. Springer, Berlin Heidelberg New York

Amthor JS (2000) The McCree–de Wit–Penning de Vries–Thornley respiration paradigms: 30 years later. Ann Bot (Lond) 86:1–20

Amthor JS (2003) Efficiency of lignin biosynthesis: a quantitative analysis. Ann Bot (Lond) 91:673–695

Austin RB (1999) Yield of wheat in the United Kingdom: recent advances and prospects. Crop Sci 39:1604–1610

Austin RB, Ford MA, Edrich JS, Blackwell RD (1977) The nitrogen economy of winter wheat. J Agric Sci (Cambridge) 88:159–167

Berry S, Rumberg B (1999) Proton to electron stoichiometry in electron transport of spinach thylakoids. Biochim Biophys Acta 1410:248–261

Björkman O, Demmig-Adams B (1994) Regulation of photosynthetic light energy capture, conversion, and dissipation in leaves of higher plants. In: Schulze ED, Caldwell MM (eds) Ecophysiology of photosynthesis. Springer, Berlin Heidelberg New York, pp 17–70

Cassman KG, Dobermann A, Walters DT, Yang H (2003) Meeting cereal demand while protecting natural resources and improving environmental quality. Annu Rev Environ Resourc 28:315–358

Cooper JP (1975) Control of photosynthetic production in terrestrial systems. In: Cooper JP (ed) Photosynthesis and productivity in different environments. Cambridge University Press, Cambridge, pp 593–621

Crafts-Brandner SJ, Salvucci ME (2000) Rubisco activase constrains the photosynthetic potential of leaves at high temperature and CO_2. Proc Natl Acad Sci U S A 97:13430–13435

Demmig-Adams B, Adams WW, III (1992) Photoprotection and other responses of plants to high light stress. Annu Rev Plant Physiol Plant Mol Biol 43:599–626

de Veau EJ, Burris JE (1989) Photorespiratory rates in wheat and maize as determined by ^{18}O-labeling. Plant Physiol 90:500–511

Dobermann A (2000) Future intensification of irrigated rice systems. In: Sheehy JE, Mitchell PL, Hardy B (eds) Redesigning rice photosynthesis to increase yield. International Rice Research Institute, Los Baños, Philippines, and Elsevier, Amsterdam, pp 229–247

Duncan WG (1971) Leaf angles, leaf area, and canopy photosynthesis. Crop Sci 11:482–485
Duvick DN (2005) The contribution of breeding to yield advances in maize (*Zea mays* L.). Adv Agron 86:83–145
Earl HJ, Tollenaar M (1998) Differences among commercial maize (*Zea mays* L.) hybrids in respiration rates of mature leaves. Field Crops Res 59:9–19
Egli DB (1998) Seed biology and the yield of grain crops. CAB International, Wallingford New York
Egli DB, Bruening WP (2003) Increasing sink size does not increase photosynthesis during seed filling in soybean. Eur J Agron 19:289–298
Ehleringer J, Pearcy RW (1983) Variation in quantum yield for CO_2 uptake among C_3 and C_4 plants. Plant Physiol 73:555–559
Evans JR (1989) Photosynthesis and nitrogen relationships in leaves of C_3 plants. Oecologia 78:9–19
Evans LT (1993) Crop evolution, adaptation and yield. Cambridge University Press, Cambridge
Evans LT, Fischer RA (1999) Yield potential: its definition, measurement, and significance. Crop Sci 39:1544–1551
Fischer RA (1983) Wheat. In: Smith WH, Banta SJ (eds) Potential productivity of field crops under different environments. International Rice Research Institute, Los Baños, Philippines, pp 129–154
Fischer RA, Rees D, Sayre KD, Lu Z-M, Condon AG, Larque Saavedra A (1998) Wheat yield progress associated with higher stomatal conductance and photosynthetic rate, and cooler canopies. Crop Sci 38:1467–1475
Hatch MD, Agostino A, Jenkins CLD (1995) Measurement of the leakage of CO_2 from bundle-sheath cells of leaves during C_4 photosynthesis. Plant Physiol 108:173–181
Irvine JE (1983) Sugarcane. In: Smith WH, Banta SJ (eds) Potential productivity of field crops under different environments. International Rice Research Institute, Los Baños, Philippines, pp 361–381
Jenkins CLD, Furbank RT, Hatch MD (1989) Mechanism of C_4 photosynthesis. Plant Physiol 91:1372–1381
Keeling CD, Whorf TP (2005) Atmospheric CO_2 records from sites in the SIO air sampling network. In: Trends: a compendium of data on global change. Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, U.S. Department of Energy, Oak Ridge [http://cdiac.esd.ornl.gov]
Kraus E (1992) The absence of a consistent relation between yield and rate of dark respiration in selected populations of perennial ryegrass. In: Lambers H, van der Plas LHW (eds) Molecular, biochemical and physiological aspects of plant respiration. SPB Academic, The Hague, pp 567–571
Ku MS, Cho D, Li X, Jiao DM, Pinto M, Miyao M, Matsuoka M (2001) Introduction of genes encoding C_4 photosynthesis enzymes into rice plants: physiological consequences. Novartis Found Symp 236:100–111
Lawlor DW (1995) Photosynthesis, productivity and environment. J Exp Bot 46:1449–1461

Leegood RC (1999) Photosynthesis in C_3 plants: the Benson-Calvin cycle and photorespiration. In: Lea PJ, Leegood RC (eds) Plant biochemistry and molecular biology, 2nd edn. Wiley, Chichester, pp 29–50

Leegood RC (2002) C_4 photosynthesis: principles of CO_2 concentration and prospects for its introduction into C_3 plants. J Exp Bot 53:581–590

Lindquist JL, Arkebauer TJ, Walters DT, Cassman KG, Dobermann A (2005) Maize radiation use efficiency under optimal growth conditions. Agron J 97:72–78

Long SP, Postl WF, Bolhár-Nordenkampf HR (1993) Quantum yields for uptake of carbon dioxide in C_3 vascular plants of contrasting habitats and taxonomic groupings. Planta 189:226–234

Loomis RS (1983) Productivity of agricultural systems. In: Lange OL, Nobel PS, Osmond CB, Ziegler H (eds) Physiological plant ecology IV. Springer, Berlin Heidelberg New York, pp 151–172

Loomis RS, Amthor JS (1996) Limits to yield revisited. In: Reynolds MP, Rajaram S, McNab A (eds) Increasing yield potential in wheat: breaking the barriers. CIMMYT, Mexico, DF, pp 76–89

Loomis RS, Amthor JS (1999) Yield potential, plant assimilatory capacity, and metabolic efficiencies. Crop Sci 39:1584–1596

Loomis RS, Connor DJ (1992) Crop ecology. Cambridge University Press, Cambridge

Loomis RS, Gerakis PA (1975) Productivity of agricultural ecosystems. In: Cooper JP (ed) Photosynthesis and productivity in different environments. Cambridge University Press, Cambridge, pp 145–172

Loomis RS, Williams WA, Duncan WG (1967) Community architecture and the productivity of terrestrial plant communities. In: San Pietro A, Greer FA, Army TJ (eds) Harvesting the sun: photosynthesis in plant life. Academic Press, New York London, pp 291–308

McCree KJ (1972) The action spectrum, absorptance and quantum yield of photosynthesis in crop plants. Agric Meteorol 9:191–216

McCree KJ (1981) Photosynthetically active radiation. In: Lange OL, Nobel PS, Osmond CB, Ziegler H (eds) Physiological plant ecology I. Springer, Berlin Heidelberg New York, pp 41–55

McCree KJ, Keener ME (1974) Effect of atmospheric turbidity on the photosynthetic rates of leaves. Agric Meteorol 13:349–357

Monteith JL (1977) Climate and the efficiency of crop production in Britain. Philos Trans R Soc Lond B Biol Sci 281:277–294

Monteith JL (1978) Reassessment of maximum growth rates for C_3 and C_4 crops. Expl Agric 14:1–5

Morrison MJ, Voldeng HD, Cober ER (1999) Physiological changes from 58 years of genetic improvement of short-season soybean cultivars in Canada. Agron J 91:685–689

Murchie EH, Chen Y, Hubbart S, Peng S, Horton P (1999) Interactions between senescence and leaf orientation determine in situ patterns of photosynthesis and photoinhibition in field-grown rice. Plant Physiol 119:553–563

Naidu SL, Long SP (2004) Potential mechanisms of low-temperature tolerance of C_4 photosynthesis in *Miscanthus* × *giganteus*: an in vivo analysis. Planta 220:145–155

Noctor G, Foyer CH (1998) A re-evaluation of the ATP:NADPH budget during C_3 photosynthesis: a contribution from nitrate assimilation and its associated respiratory activity? J Exp Bot 49:1895–1908

Pänke O, Rumberg B (1997) Energy and entropy balance of ATP synthesis. Biochim Biophys Acta 1322:183–194

Parry MAJ, Andralojc PJ, Mitchell RAC, Madgwick PJ, Keys AJ (2003) Manipulation of rubisco: the amount, activity, function and regulation. J Exp Bot 54:1321–1333

Penning de Vries FWT (1975) The cost of maintenance processes in plant cells. Ann Bot (Lond) 39:77–92

Penning de Vries FWT, Brunsting AHM, van Laar HH (1974) Products, requirements and efficiency of biosynthesis: a quantitative approach. J Theor Biol 45:339–377

Penning de Vries FWT, van Laar HH, Chardon MCM (1983) Bioenergetics of growth of seeds, fruits and storage organs. In: Smith WH, Banta SJ (eds) Potential productivity of field crops under different environments. International Rice Research Institute, Los Baños, Philippines, pp 37–59

Poirier Y (1999) Production of new polymeric compounds in plants. Curr Opin Biotechnol 10:181–185

Portis AR, Jr (2003) Rubisco activase – rubisco's catalytic chaperone. Photosynthesis Res 75:11–27

Radin JW, Lu ZM, Percy RG, Zeiger E (1994) Genetic-variability for stomatal conductance in pima cotton and its relation to improvements of heat adaptation. Proc Natl Acad Sci U S A 91:7217–7221

Regierer B, Fernie AR, Springer F, Perez-Melis A, Leisse A, Koehl K, Willmitzer L, Geigenberger P, Kossmann J (2002) Starch content and yield increase as a result of altering adenylate pools in transgenic plants. Nature Biotechnol 20:1256–1260

Reynolds MP, van Ginkel M, Ribaut J-M (2000) Avenues for genetic modification of radiation use efficiency in wheat. J Exp Bot 51:459–473

Richards RA (2000) Selectable traits to increase crop photosynthesis and yield of grain crops. J Exp Bot 51:447–458

Sage RF, Reid CD (1994) Photosynthetic response mechanisms to environmental change in C3 plants. In: Wilkinson RE (ed) Plant–environment interactions. Marcel Dekker, New York, pp 413–499

Santos MJD, Wigdorovitz A (2005) Transgenic plants for the production of veterinary vaccines. Immunol Cell Biol 83:229–238

Scheller J, Conrad U (2005) Plant-based material, protein and biodegradable plastic. Curr Opin Plant Biol 8:188–196

Seybold A (1933) Über die optischen eigenschaften der laubblätter. IV. Planta 21:251–265

Sharma-Natu P, Ghildiyal MC (2005) Potential targets for improving photosynthesis and crop yield. Curr Sci 88:1918–1928

Sharp RE, Matthews MA, Boyer JS (1984) Kok effect and the quantum yield of photosynthesis. Plant Physiol 75:95–101

Sheehy JE (2000) Limits to yield for C_3 and C_4 rice: an agronomist's view. In: Sheehy JE, Mitchell PL, Hardy B (eds) Redesigning rice photosynthesis to increase yield. International Rice Research Institute, Los Baños, Philippines, and Elsevier, Amsterdam, pp 39–52

Shibles RM, Weber CR (1965) Leaf area, solar radiation interception and dry matter production by soybeans. Crop Sci 5:575–577

Shinano T, Osaki M, Komatsu K, Tadano T (1993) Comparison of production efficiency of the harvesting organs among field crops. I. Growth efficiency of the harvesting organs. Soil Sci Plant Nutr 39:269–280

Sinclair TR, Sheehy JE (1999) Erect leaves and photosynthesis in rice. Science 283:1455

Smidansky ED, Clancy M, Meyer FD, Lanning SP, Blake NK, Talbert LE, Giroux MJ (2002) Enhanced ADP-glucose pyrophosphorylase activity in wheat endosperm increases seed yield. Proc Natl Acad Sci U S A 99:1724–1729

Smidansky ED, Martin JM, Hannah LC, Fischer AM, Giroux MJ (2003) Seed yield and plant biomass increases in rice are conferred by deregulation of endosperm ADP-glucose pyrophosphorylase. Planta 216:656–664

Smith WH, Banta SJ (eds) (1983) Potential productivity of field crops under different environments. International Rice Research Institute, Los Baños, Philippines

Spano G, Di Fonzo N, Perrotta C, Platani C, Ronga G, Lawlor DW, Napier JA, Shewry PR (2003) Physiological characterization of 'stay green' mutants in durum wheat. J Exp Bot 54:1415–1420

Spreitzer RJ, Salvucci ME (2002) Rubisco: structure, regulatory interactions, and possibilities for a better enzyme. Annu Rev Plant Biol 53:449–475

Stoger E, Ma JK-C, Fischer R, Christou P (2005) Sowing the seeds of success: pharmaceutical proteins from plants. Curr Opin Biotechnol 16:167–173

Tollenaar M, Wu J (1999) Yield improvement in temperate maize is attributable to greater stress tolerance. Crop Sci 39:1597–1604

Uemura K, Anwaruzzaman, Miyachi S, Yokota A (1997) Ribulose-1,5-bisphosphate carboxylase/oxygenase from thermophilic red algae with a strong specificity for CO_2 fixation. Biochem Biophys Res Commun 233:568–571

Wang Q, Zhang QD, Zhu XG, Lu CM, Kuang TY, Li CQ (2002) PSII photochemistry and xanthophyll cycle in two superhigh-yield rice hybrids, Liangyoupeijiu and Hua-an 3 during photoinhibition and subsequent restoration. Acta Botanica Sinica 44:1297–1302

Wang QA, Lu CM, Zhang QD (2005) Midday photoinhibition of two newly developed super-rice hybrids. Photosynthetica 43:277–281

Warren Wilson J (1967) Ecological data on dry-matter production by plants and plant communities. In: Bradley EF, Denmead OT (eds) The collection and processing of field data. Wiley, New York London Sydney, pp 77–123

Watson DJ (1958) The dependence of net assimilation rate on leaf area index. Ann Bot (Lond) 22:37–54

Whittaker RH (1975) Communities and ecosystems, 2nd edn. Macmillan, New York

Wilson D (1975) Variation in leaf respiration in relation to growth and photosynthesis of *Lolium*. Ann Appl Biol 80:323–338

Wilson D (1982) Response to selection for dark respiration rate of mature leaves in *Lolium perenne* and its affect on growth of young plants and simulated swards. Ann Bot (Lond) 49:303–312

Zhu X-G, Portis AR Jr, Long SP (2004) Would transformation of C_3 crop plants with foreign rubisco increase productivity? A computational analysis extrapolating from kinetic properties to canopy photosynthesis. Plant Cell & Environ 27:155–165

3

Marker assisted selection and genomics of industrial plants

Giuseppe Mandolino

C.R.A. - Istituto Sperimentale per le Colture Industriali, Via di Corticella 133, 40128 Bologna, Italy (e-mail: g.mandolino@isci.it)

1. Introduction

Though by far less popularly publicized than the 50th jubilee of the determination of DNA structure, celebrated three years ago, 2006 will be the 40th anniversary of the introduction of molecular markers in the genetic analysis, by Richard Lewontin in 1966. From the earliest, labour intensive and time-consuming applications to the study of the natural populations, to the massive exploitation in genome mapping of important plant species, it took therefore less than half a century for molecular markers to become a fundamental tool of theoretical and applied genetics. The oldest method for the analysis of the animal and plants' genomes is their mapping, i.e. the ordered positioning of a number of tags, acting as markers, along the entire length of the genome itself or, in the case of the eukaryotic genomes, of each of the chromosomes in which it is fragmented. The concept that two phenotypic traits can be inherited more often together rather than separately, probably dates back to the early breeding experiences. In the early decades of last century, the developments of genetic analysis led to the construction of the first genetic maps, consisting of a few tens of markers – mostly phenotypic, visible or easily scorable traits (Sturtevant 1913). It took about 50 years for a new breakthrough in the linkage mapping technologies and strategies to occur, as it was recognized that molecular tags, for example the isoenzymatic variants coded by different alleles at the

P. Ranalli (ed.), Improvement of Crop Plants for Industrial End Uses, 59–82.

same genetic locus, could be treated as markers as well (Lewontin and Hubby 1966), and their association with other traits, or between them, could be studied by standard genetic means.

The previously existing linkage maps, filled with only a limited number of phenotypic markers and genes characterized by segregation ratios, were rapidly enriched by the introduction of isoenzymatic markers. The following revolutions, the use of a wide choice of DNA-based markers for genetic analysis (Botstein 1980), only required 15 years after the introduction of isoenzymes, and led after a few more years to the early highly saturated human, animal and plant linkage maps. At the mid-Nineties, the maps of the major crops, including several important industrial ones (e.g. potato, sugarbeet) were densely studded with several markers, obtained with different molecular technologies, and ordered by detailed genetic analysis. Marker technology was an important tool for either positioning and tagging genes or QTLs on specific chromosome regions, and exploiting them in the marker assisted selection (MAS). It seemed to occur a strict inverse relationship between the time required to develop a new marker type, and the number of markers that each new introduction made available to the geneticists and breeders. The limit such an acceleration pointed at, was soon reached: the collapse in the cost of DNA sequencing and equipment, and the development of automatic, high-throughput technologies made available, in a rapidly increasing number of species, the ultimate marker, i.e. the gene or even the whole genomic sequences, in draft versions constantly refined and annotated; it has been a matter of only a short time from the publication of the human genomic sequence, and the complete sequencing of a model plant, the thale cress (The Arabidopsis Genome Initiative, 2000). Soon the genomes of other plants were sequenced: rice, tomato, poplar. Several international initiatives and research consortia will lead in the very next future to the availability of the entire sequence of other crop plants, though these last years made increasingly evident, for the full exploitation of the data available, the necessity of the functional annotation and characterization of the expressed and regulative sequences available (see Chapter 1 of this book).

Industrial crops were fully part of these development, and the advancement of the research in the area of marker-assisted breeding and genomics, depended on the rapid developments of the technologies and strategies necessary, and on the economic relevance of the crop, leading to more or less extensive gathering of research institutions into consortia engaged in the sequence production and/or characterization.

Some plant species, that were traditionally considered food or forage crops, can today be added to the list of typically industrial crops. Among these there are plants that can now be exploited as biomass or biofuel sources, such as sorghum or rapeseed. These new, industrial uses of

traditional forage, food or oil crops, can take further impulse from the rapidly-growing amount of genomic data accumulated since 2000. Among the several industrial crops for which highly dense genetic maps, and an high amount of genomic and functional genomic data are being accumulated, there are rapeseed, sugarbeet and hemp. These three crops are somehow paradigmatic of the evolution, along with the genomic technologies, of their end-use, and of the possible exploitation, at different levels of development, of the genomic databases. Besides their traditional use (food oil, sugar and fibre), requiring completely different industrial plants, in the latest years these three crop species can all be included in the list of actual or potential crops grown for biofuels, a rapidly expanding field of investments in Europe and America. In the present review, we will highlight some of the main advancements of the genomics and marker-assisted selection in these three industrial crop species.

2. Rapeseed

Rapeseed (*Brassica napus*) cultivation experienced a great expansion in the latest years, and it is today one of the leading oil crops in the world, along with soybean and palm. FAO reports a worldwide increase in the last years from 25 to 27 millions ha since 2000, with a 14% increase in production (FAOSTAT). It is likely that these increases were at least partially due to the fact that, along with the traditional uses of rapeseed, for animal feeding and vegetable oil, in the latest years the biodiesel option has been favored by public and private investments, and by specific legislative actions in several countries. Due to its long-lasting economic importance in both developed and developing countries, the state of the art of molecular markers development and genomics in this plant is highly advanced. Already in 1991, a genetic map essentially based on expressed cDNA probes, and covering the 19 linkage groups of the amphidiploid species *Brassica napus* was published, using as parentals of the mapping F2 population two "canola" varieties (Landry et al. 1991). Even in this early map, based on poorly polymorphic probes, it was possible a first assessment of the structure of the rapeseed genome, identifying duplicated regions and rearrangements of the duplicated loci, altering their linear order in different genome areas. Besides rapeseed, other Brassicaceae were thereafter extensively studied by means of molecular markers, revealing a substantially high degree of polymorphisms, detectable especially by DNA markers. Already in the mid-Nineties, it was possible to compare a number of maps for several different species belonging to the "U triangle" (U, 1935), with the only exception of *Brassica carinata* (Cheung and Landry 1996; Lagerkrantz and Lydiate 1996). The availability of the earliest maps in some of

these species, and the extensive use of RFLP and cDNAs deriving from studies on the model species *Arabidopsis thaliana*, started to pay off very soon, driving and accelerating the advancements of genomic research in all Brassicaceae (Teutonico and Osborn 1994). The following studies confirmed that an interesting feature of Brassica maps was the high number of rearrangements found, a feature that allowed to date with great accuracy the divergence of the different members of the family from Arabidopsis (Lagerkrantz 1998). The use of molecular markers in this plant family is therefore a good example of how molecular maps, besides their potential in marker-assisted breeding, have an immediate application to the study of the structure and evolution, natural or through domestication, of the plant families. The colinearity found between the different rapeseed maps and those obtained for other Brassicaceae, was almost complete, and their comparison was fully in support of the postulated relationships between the diplod and amphidiploid members of the family (Paterson et al. 2000; Lydiate et al. 1993; Pradhan et al. 2003). Besides, specific bioinformatics tools were developed for the systematic comparison of their genomes with that of the fully sequenced Arabidopsis: the comparative analysis of the *B. napus* genome led to the identification of 21 conserved units that can be traced back to the Arabidopsis genome, modified by duplication and arrangements (Parkin et al. 2005). The genomes within the Brassicaceae family are comparatively large or even, in the case of the amphidiploid, very large. It is of great importance the availability, for their genetic analysis, of a much smaller, densely mapped and entirely sequenced genome of a close relative with a smaller and simpler genome, such as Arabidopsis. The informations and the functional annotations stored in databases like TAIR (The Arabidopsis Information Resource, www.arabidopsis.org; see also Chapter 1) are useful short cuts for the identification of *B. napus* candidate genes (see for example Brunel et al. 1999, where also polymorphisms discriminating different *B. napus* lines were detected).

As for the exploitation of molecular markers, a particularly useful approach was developed by the Brassica Microsatellite initiative, a public-private consortium that developed over 2,000 microsatellite markers for *B. napus* genetic analysis. Several genome-specific microsatellites (i.e. specific for the A or C genome of the amphidiploid) were identified; besides, a number of highly polymorphic and reliable SSR loci for each linkage group were made publicy available, allowing the research community to exploit them for placing in "frame" any given new marker or trait in its unambiguously defined linkage group (Lydiate and Sharpe 2003). SSR markers were also largely used in rapeseed for studying their genetic diversity and the relationships between the different gene pools (Hasan et al. 2006), and high-throughput systems were devised, involving multiplex PCR

reactions and fluorescence-based detection of the fragments, for genotyping and pedigree analysis (Mitchell et al. 1997).

Finally, it should be pointed out that some members of the Brassica family were used to develop SRAP (Sequence Related Amplified Polymorphisms) markers; these markers are especially designed to target coding sequence in the genome. They are based on two 17 or 18 bp primers, one consisting of a 14 bp "core" sequence composed of a 11 bp long "filler" and a CCGG short tail, while the other primer has an AATT sequence. In both primers, three selective nucleotides are at the end of the sequence. In Brassica species, this type of multi-locus marker proved to be an effective strategy to amplify sequences particularly rich in coding regions; in fact, the CCGG sequence "targets" the exons of ORFs, particularly rich in G+C, while, at the opposite end, AATT sequence targets the AT-rich regions at the 3' ends of the genes. Upon sequencing of the polymorphic bands, it was shown that for most of them, many highly similar sequences in the Gene Bank were found following BLAST analysis. SRAP markers can be relatively easily converted to codominant ones, and they were found to have a good genome coverage in *B. oleracea*, where a combined AFLP-SRAP map was developed (Li and Quiros 2001). SRAP markers are also being tested, along with other more traditional marker systems, for marker-assisted selection of important traits in the rapeseed genetic improvement. Among the traits under selection with the aid of these markers, there is the identification of resistance genes; particularly relevant are the attempts of marker-assisted pyramiding of the different sources of blackleg resistance; white rust resistance, another resistance trait important to introgress into Indian mustard (*B. juncea*; Somers et al. 2002). As for partial resistance to *Sclerotinia sclerotiorum*, a mixed RFLP, AFLP, SSR and RAPD map was developed, and QTLs accounting for a large proportion of the variation were identified and mapped (Zhao and Meng 2003).

Finally, markers have also been exploited for the tagging of the genes involved in the biosynthesis of glucosinolates, the main secondary metabolite produced by all members of the Brassicaceae family (Li and Quiros 2001; Mahmood et al. 2003); also for the putative identification of the genes involved in their biosynthesis, it was extremely useful the candidate gene approach, based on the knowledge of the homologous genes of *A. thaliana*. By this approach, several genes involved in the synthesis and degradation of glucosinolates in different Brassicaceae, have been isolated and characterized (Barth and Jander 2006; Gao et al. 2004; Mahmood et al. 2003).

3. Sugarbeet

On February 2006, the European Council of the Ministers gave green light for the reform of the OCM sugar. The immediate consequence was a cut of the sugarbeet production in all European countries, ranging from 5% for Germany, up to 50% for Italy. One of the consequences of the reduction of the production of sugar from beets has been the necessity, for many European governments, to provide incentives for alternative uses of both the sugar factories (e.g. converted to the production of biodiesel or energy from biomasses), while for the sugar beet crop, one of the possible pathways of utilization of the sucrose was the conversion to ethanol, for biofuel production. However, for this relatively new end-use, sugar beet does not appear competitive with other crops, and the sugar beet research community will probably focus its attention on the exploitation of the biotechnology and breeding strategies for increasing sugar beet yield and quality, through the introduction of resistances to abiotic and biotic stresses, bolting and retrogradation, still a major cause of economic losses in many areas.

Sugarbeet belongs to the Chenopodiaceae family, and all the cultivated forms (sugar, chard, fodder and red beet) belong to the same species, *Beta vulgaris* ssp. *vulgaris*, included in the Section *Beta* of genus Beta (Lange et al. 1999). In the same species is also included a distinct subspecies, perfectly intermating with all the cultivated forms, *Beta vulgaris* ssp. *maritima* (the sea beet). Populations of this wild subspecies exist along most of the coastal areas of Europe, and have been repeatedly exploited in sugarbeet breeding, as they often are source of resistances to cercospora leaf spot and rhizomania, two of the major diseases causing severe production losses (Stevanato et al. 2001; Biancardi et al. 2002). The three remaining Sections of the genus Beta: *Nanae*, *Corollinae* and *Procumbentes*, only include wild species (e.g. *B. nana*, *B. patellaris*, *B. procumbens*, *B. webbiana*). In these Sections, other important sources of resistance to cercospora and to the nematode *Heterodera schachtii* exist (Luterbacher et al. 2005; Speckmann and de Bock 1982; Heijbroek et al. 1983), though their introgression in the cultivated gene pool is more difficult, due to high incompatibility with the *B. vulgaris* species. Among the earliest applications of molecular markers to Beta studies, there has been the comparison of the cultivated and wild germplasm, and the characterization of the ancestral or actual gene flow occurring between them. Isoenzymes, RFLP and cDNA probes were used to detect polymorphisms, to establish phylogenetic relationships between the different Beta taxa, and to fingerprint interspecific hybrids or addition or translocation lines obtained from crosses between incompatible *B. vulgaris* and *B. procumbens* or *B. patellaris* (Oléo et al. 1986; Nagamine et al. 1989). In these early works, genomic RFLPs were

mainly used, but the detection of polymorphisms between sugar beet varieties was quite low, at least until the use of hypervariable "minisatellite" probes, targeting several different loci in one single hybridization step, was introduced (Jung et al. 1993). These works confirmed the strict proximity of the cultivated beet gene pool with the sea beet, while other species, belonging to different Sections of the genus Beta, were much more distantly related, based on Nei and Li similarity coefficients: it was reported that only 34% of the RFLP probes used cross-hybridized between these species (Jung et al. 1993). Molecular markers were extensively used for detailed studies of the gene flow within wild *B. vulgaris* ssp. *maritima* populations (Raybould et al. 1996; Tufto et al. 1998), and particular relevance assumed the studies of the gene flow (as estimated by AFLP or RFLP markers) between the bolting or flowering transgenic sugar beets, and the wild or weedy populations. In these studies, the risk of the transgene escape from cultivated fields was assessed (Desplanque et al. 1999), and the genetic erosion of the wild beet populations was evaluated (Bartsch et al. 2002). Gene flow studies, and their impact on the risk assessment protocols and on the legislations regulating the introduction of transgenic varieties, were indeed one of the main driving force for the discovery and application of several microsatellite markers in the Beta genus (Cureton et al. 2002; Richards et al. 2004). Although, strictly speaking, this research field is not real marker-assisted selection, it can be considered as a new field of application, a "marker-assisted risk assessment" strategy, that will probably play an increasingly important role in a number of cases of decision-making processes related with the introduction of transgenic varieties and their coexistence with traditional crops.

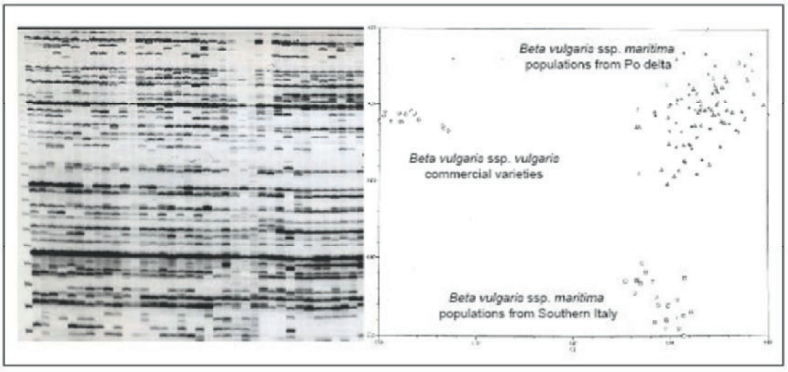


Fig. 3.1. AFLP patterns (left) and principal coordinate analysis of AFLP data (right) for varieties and wild populations of *Beta vulgaris* (ssp. *vulgaris* and *maritima*).

The existence in Beta of some Section-specific genomic probes, corresponding to repeated, satellite sequences, had far-reaching consequences. In fact, since 1990, probes hybridizing specifically to satellite DNA of members of the Procumbentes Section were used in the marker-assisted identification of the sugarbeet lines carrying the monosomic addition from *B. procumbens*, enclosing the resistance gene to *Heterodera schachtii* (Schmidt et al. 1990; Salentijn et al. 1992). These Section-specific markers were used to "bracket" the resistance gene, and then to clone it (Klein-Lankhorst et al. 1994; Cai et al. 1997). Marker-assisted tagging of specific chromosome segments was also carried out in screenings of monosomic additions and translocation lines from interspecific crosses (*B. vulgaris* x *B. procumbens* or *B. vulgaris* x *B. corolliflora*), for the isolation of other sources of nematode resistance (Heller et al. 1996; Kleine et al. 1998; Gao et al. 2001).

Rhizomania is another important disease for which sugar beet breeding has reached, in the last 15 years, several successes. The introgression of the resistance trait appears the main strategy to obtain high yields and root quality in the rhizomania-infected soils (Asher 1993; Biancardi et al. 2002). The breeding work took advantage of the availability of greenhouse tests based on a positive correlation between rhizomania resistance and virus content, as evaluated by ELISA assay of the roots of artificially-infected plantlets. This test was therefore the basis of many breeding programs, but was also useful for establishing an association between the various resistance sources, and the different linkage groups and markers in different mapping populations. The "Holly type" resistance was mapped in the linkage group IV, with an RFLP marker about 7 cM from *Rr1* gene (Barzen et al. 1992); bulk segregant analysis (BSA; Michelmore et al. 1991) was used to screen for markers associated to this same locus, and RAPD and SCAR markers were obtained (Barzen et al. 1997). Interestingly, these markers also were able to identify an apparently different source of resistance, originating from *B. vulgaris* ssp. *maritima*, and introgressed in the sugar beet line R104 (Biancardi et al. 2002). Further research, also based on map comparison and the construction of consensus maps (Schumacher et al. 1997), revealed that another associated resistance, deriving from a further *B. vulgaris* ssp. *maritima* accession, WB42, was associated but distinct, mapping at a different locus called *Rz2*, to keep it distinct from the Holly/R104 one (called *Rz1*; Scholten et al. 1999). In the case of rhizomania, molecular markers, therefore, were not only useful for marker-assisted selection (with the limit that most of the available markers are protected by proprietary rights, as they have been developed by private companies), but also contributed important insights on the long-debated issue of the relationships between the different resistance sources (Biancardi et al. 2002). Efforts were also made to combine different linkage

maps from different populations, each contributing specific resistance or quality traits, and segregating for QTL for yield-related traits (Weber et al. 1999; 2000). However, more recently the potential for marker-assisted selection for yield and quality has also been explored by a functional genomic approach (see below).

As for the markers for disease resistances, a different approach recently applied to sugar beet illustrates well the spreading of non-mapping strategies, made possible by the increasingly wide number of expressed sequences available in public repositories. Sequencing of resistance genes against several pathogens and in several plant species, made clear that shared domains exist. Therefore, it has been possible to design primers and to isolate resistance gene analogues, determining their functional characteristics, mapping them on the existing linkage maps, and associating them to mapped resistance loci, as it has been the case for rhizomania and cercospora leaf spot resistance (Hunger et al. 2003). This approach can be particularly promising for identification of candidate genes for the resistance to *Cercospora beticola*, the causal agent of cercospora leaf spot; this resistance trait showed in selection programs a slow rate of gains, as it is typical of quantitative traits, and it is inversely correlated with the yield parameters (Skaracis and Biancardi 2000). Difficulties in the development of effective markers for this trait are due to problems in exploiting a reliable *in vitro* method for resistance evaluation, and on the number and chromosomal location of QTLs associated, that are different in different studies, and even different depending upon the inoculation conditions (artificial conditions or naturally infested fields) (Weiland and Koch 2004).

Among the several sugar beet linkage maps published, a special mention deserves the one from Pillen et al. (1993), as this map includes evidences of close association (and therefore, possible exploitability in the breeding) with the fertility restoration gene, a very important trait in the development of O-type lines. These latter are plants capable of "maintaining" the male sterility, and therefore essential for the multiplication of the male sterile lines, in turn used as parental for hybrid seed production. The "restoration of fertility" locus, at which maintainer lines are endowed with the recessive allele x, was mapped terminally on linkage group III, about 9 cM from an RFLP marker; however, it should be pointed out that current genetical models suggest the existence of two distinct loci for fertility restoration (X and Z), on two different linkage groups. The importance of this trait in the sugar beet industry is confirmed by the amount of studies carried out in the last ten years, often with markedly different approaches. QTLs were described on linkage group III and IV in segregating populations screened by RFLP markers, and explaining from 72 to 79% of the variation observed; this analysis suggested the existence of minor alleles in all mapping populations (Hjerdin-Panagopoulos et al. 2002). In another study, BSA was

used, and several AFLP and RAPD markers were identified, three of which were confirmed to co-segregate with the restoration of fertility (Hagihara et al. 2005). Despite these successes, the gene(s) responsible of the maintaining phenotype have not yet been cloned, their functional analysis is still to come, and details of the interactions of nuclear gene products with the cytoplasmic-encoded male sterility determinants have not yet been clarified for sugar beet.

Despite the transition from genetic to genomic analysis has been in sugar beet less advanced than in other crop species, huge collections of expressed genes have been deposited in the gene banks (e.g. TIGR, www.tigr.org; The Sugarbeet EST Database http://genomics.msu.edu/sugarbeet/) and are presently being screened for functional analysis and for genes possibly correlated to agronomically valuable traits. The progressive accumulation of sequence informations on sugar beet was exploited for the enrichment of the existing genetic maps with SNPs, or for the fingerprinting of sugar beet genotypes. Particularly interesting is the approach linking fragments of expressed genes, often of some relevance from the industrial point of view, and the SNPs found in sugar beet germplasm within these sequences, and anchored to the existing molecular maps (Schneider et al. 1999). SNPs were also identified in sugar beet by gel-based assays, such as single strand conformation polymorphisms (SSCP) and heteroduplex analysis (HA) (Schneider et al. 2001); the markers developed in this way proved to have a polymorphism information content (PIC) around 0.47, not as high as SSR, but superior to AFLP or RFLP markers. Sugar beet SNPs identified by these approaches could also be located in the different linkage group; multiplex assays were developed to fingerprint genotypes simultaneously at different loci in single PCR reactions for each linkage group, and to detect these polymorphisms by fluorescence in capillary electrophoresis, in a high-throughput, semi-automated system (Möhring et al. 2004).

In general, these examples show that marker-assisted selection is at a quite mature stage in sugar beet, for different industrially important traits. Besides, in the last few years, sugar beet functional genomics took advantage of several specific international projects and cooperation's, and it is likely that in the next future the number of disease resistance, quality and yield traits, for which the gene(s) and alleles responsible for the variation are completely characterized, will substantially increase.

4. Hemp

Hemp, *Cannabis sativa*, is probably one of the most ancient non food crops cultivated by mankind. Hemp is endowed of a particularly oil-rich

seed, and of species-specific secondary compounds accumulating in the inflorescences (cannabinoids); because of these characteristics, hemp has been considered a candidate for biofuel production and pharmaceutical applications respectively; besides, a number of novel uses for its fibre have been developed (e.g. in paper and automotive industry). Several initiatives and industrial enterprises, aimed at demonstrating the feasibility and sustainability of such uses, and the consequent worth of the re-discovery of this ancient crop also for more modern industrial uses, have been carried out, often with good commercial success (Guy and Stott 2004).

The first concept that is important to stress, when dealing with this typically industrial species, is that most taxonomists recognize the existence of a single species in the Cannabaceae family, namely *C. sativa*. Therefore, the distinction between *C. sativa* as a fibre crop -deserving sustain for its beneficial added values such as low chemical inputs, eradication of some soil-borne pathogens, potentially complete utilization of all its parts, etc.- and *C. indica* as an illegal species, genetically different from the first, but phenotypically undistinguishable from it, and only suited for drug abuse, should be overcome. The two forms of Cannabis, that can be at most considered different subspecies, but more correctly different races or varieties (chemotypes: see below), are perfectly interfertile, and they should be considered belonging to a single gene pool (Small et al. 1976). However, the recent history of hemp cannot be made without considering two facts: first, forms of *Cannabis sativa* L., endowed with levels of a single secondary compound, Δ^9-tetrahydrocannabinol (THC), above 0.30% of the inflorescence dry weight, are considered illegal (and not eligible for EU subsidies) and cannot be cultivated; and second, that the close relatedness of the tradition-revivifying, environmentally sustainable and agronomically beneficial fibre hemp with the marihuana, has been used by opinion groups as an argument in favor of its liberalization. As an obvious consequence, far from limiting itself to industrial and technical issues, the question of hemp cultivation in Europe and America has been strongly conditioned by the analytical and legislative issues, in most cases hampering the re-discovery and diffusion of this plant for a number of industrial exploitations (Ranalli et al. 1999; Karus and Vogt 2004; Guy and Stott 2004). A further consequence of this paradox was that forensic scientists, rather than breeders, set up the basic knowledges leading to the exploitation of molecular markers in Cannabis research (see Mandolino and Carboni 2004, for a review of forensic applications). Basically, these early applications aimed at the development of methods recognizing the presence of the illicit "cannabis" plant material, distinguishing it from other plant sources, and at the analysis of the variability within illegal plant material, to reconstruct phylogenies of the different drug strains, and the routes of their diffusion. For example, a short intergenic sequence located between the chloroplast genes for the

LEU and PHE transport RNAs can act, upon amplification, as a marker for the recognition of *Cannabis sativa* DNA (Wilkinson and Linacre 2000). The internal transcribed spacers I and II (ITS1 and ITS2) of the nuclear ribosomal genes were used in forensic applications to univocally identify *Cannabis sativa* (Siniscalco Gigliano et al. 1997).

These examples made confirm the fact that forensic research had a major role in the development of molecular markers in Cannabis research: AFLP analysis specific for marihuana samples (Coyle et al. 2003), and RAPD (Gillan et al. 1995; Jagadish et al. 1996) and ISSR (Kojoma et al. 2002) markers were used in attempts to establish a direct relationship between the cannabinoid type of the plant, and the markers identified. However, no correlations between any specific markers and the gas-chromatographic or HPLC cannabinoid profiles of different Cannabis chemotypes were identified in these studies, and the development of chemotype-specific markers has only recently been achieved (see below).

Microsatellites markers were described and applied to the study of Cannabis population and their variability only very recently (Alghanim and Almirall 2003; Gilmore and Peakall 2003; Hsieh et al. 2003). In hemp, the most common repeated motif is the dinucleotide GA/CT; di- and trinucleotide repeats were the most frequent, with an allele number ranging from 2 up to 28 (Hsieh et al. 2003). These markers were useful in describing the genetic relatedness of the Cannabis accessions considered, but no marker associated with the chemotype, and suitable for marker-assisted marijuana identification were found (Mandolino and Carboni 2004).

Variation is a key feature of *Cannabis sativa*, and this makes its fingerprinting and mapping essentially a statistical task. SSR and RAPD markers were used for genotyping, and the variability found analyzing varieties, populations and accessions was very high; in one study, out of 93 plants examined by SSRs, only four (belonging to the same drug accession) were not distinguishable from each other (Gilmore et al. 2003), and also RAPD markers showed reproducibly high levels of variation (Faeti et al. 1996). Therefore, a first level of analysis is variation assessment within varieties, populations or accessions, and between them; other levels of analysis are then possible (between and within chemotypes, sexes, etc.). Analysis of Molecular Variance (AMOVA, Excoffier et al. 1992) has been a widely used tool for analysis of marker variation, especially in natural and allogamous populations. In Cannabis, the majority of the observed marker variation (from 50 to 73%) was attributable to individual differences within accessions (Forapani et al. 2001; Gilmore and Peakall 2003). If the accessions are roughly divided into the two groups “drug” and “non drug” (chemotype I and III, see below), only 6% of the variation was attributable to the chemotype: it was concluded that there was "no clear split or defined boundary between drug and non drug materials". Conceptually similar

results had been obtained also by Forapani et al. (2001), that confirmed the very high degree of variation within the cultivars or accessions previously identified, ranging from 31% of polymorphic loci in an inbred female Cannabis strain, up to 78% in Fibranova, a cross-bred fibre variety. Forapani et al. (2001) also found that the majority (66%) of the markers had a calculated F_{ST} value below the average (0.48); the authors interpreted this finding as a confirmation of the existence of a widely shared gene pool in Cannabis, with limited cultivar boundaries and relatively poor loci segregation between different populations.

The marker variation observed was exploited to construct the few available molecular maps of *Cannabis sativa*, including RAPD or AFLP markers (Carboni et al. 2000; Flachowski et al. 2001; Mandolino and Ranalli 2002), but lacking until now agronomically relevant traits, like fibre content or quality, or monoecy; the genetic basis of these traits are indeed still poorly understood. The available maps still have limited markers density, and no attempts to define anchor markers, or to establish relationships between the molecular maps and the Cannabis chromosomes has been done yet, with the only exception of the Y chromosome and of the male-associated markers (see below). The extremely high variability found is a general problem in many allogamous crop species, but it might reveal particularly important in hemp.

Sex and monoecy are particularly important traits to score during the selection programs for industrial hemp. Dioecious varieties, made of male and female plants, are generally high-yielding, more disease-tolerant, but less uniform (a strong sexual dimorphism is present) and less amenable to mechanical harvesting than monoecious varieties. This latter type of varieties (presently the most diffused and cultivated in Europe) are made of variable proportions of female and monoecious plants, have high seed yields (because all plants contribute to seed formation), high habitus uniformity (favoring mechanical harvesting), but a narrower genetic base (due to the selective pressure necessary to maintain the monoecious trait in a significant proportion of the plants), a lower biomass yield, and the necessity of strict isolation and seed batch control during seed multiplication, due to the lower competitiveness of monoecious pollen compared with pollen from contaminating male (dioecious) plants. The practice of hemp breeding sometimes requires the identification of the sexes (male, female, monoecious), with the male sex determination being especially important for the different strategies of improvement in dioecious hemp, and for seed quality controls in monoecious hemp. Early identification of the male plants is today an example of completely effective and 100% reliable marker-assisted selection. In fact, since 1999, strictly male-specific (probably Y-chromosome located) molecular markers were described by several groups, obtained by RAPD markers (Mandolino et al. 1999), then

transformed in SCAR markers upon sequencing of the discriminating fragments (Mandolino et al. 1999; Mandolino et al. 2002); also AFLP and SSR sex-specific markers were later described (Peil et al. 2003; Rode et al. 2005). In most cases, the strict association to the male phenotype was attributable to the localization of the marker on the region of the Y chromosome excluded from recombination with homologous areas of the X chromosome (Mandolino et al. 2002), while in another case the marker was located on the Y chromosome by direct FISH analysis (Sakamoto et al. 2000). In all cases reported where these male-specific markers have been sequenced, a high similarity between them and with LINE-like retrotransposons was reported (Mandolino et al. 1999; Sakamoto et al. 2000; Sakamoto et al. 2005).

The marker named $SCAR_{400}$, described in Mandolino et al. (1999), was routinely used in selection programs for both dioecious and monoecious hemp, demonstrating its full potential and association; direct PCR amplification protocols of tiny tissue fragments were devised, simplifying the scoring of the male sex in a huge number of samples (Mandolino and Ranalli 2002); this marker was tested and used also by commercial seed companies with excellent results.

One peculiar exploitation of sex-linked markers, came from the possibility to score at an extremely early stage of differentiation the genetically determined male plants, well before any sex-related structure was visible. This opportunity allowed the study of genome-wide gene expression at very early stages of the sexual determination in male and female sexes. By this "marker-assisted" gene expression analysis, it was possible to identify by cDNA-AFLP and clone some sequences differentially expressed in male and female plants (Moliterni et al. 2004).

Monoecy is another very important character for which a marker system would be useful. However, the above described male-specific marker is also suitable for keeping under strict control the “contaminating” male plants in a monoecious stand or seed lot. The exploitation of this marker could allow the introduction, by the seed companies commercializing monoecious seed, of the “elite” or “superelite” terms for seed batches, depending on the number of contaminating males expected (Mandolino and Carboni 2004). This marker can be therefore already be exploited both in MAS and in seed quality certifications of monoecious varieties. Monoecy and flowering time are today among the main targets for genetic analysis and marker-assisted breeding; the availability of male- and of monoecious-specific markers, possibly combined in a single PCR assay, would allow the complete identification of the sexual phenotype of plants belonging to any variety or breeding lines.

As anticipated at the beginning of this section, one of the main targets of modern industrial hemp breeding is the early identification of the

chemotype, i.e. of the cannabinoid(s) a given genotype is endowed with. Today, the determination of the chemotype and of the absolute amounts of each cannabinoid is carried out by labor-intensive gas-chromatography or HPLC; such analysis is mandatory to get the EU subsidies by industrial hemp growers: THC levels in a sample of plants of the stand must be below 0.30% of the inflorescence dry weight. Though the necessary techniques are susceptible of being speeded up and automated, the development of specific molecular markers predictive of the type and (ideally) amount of each of the cannabinoids a plant can manufacture, would be very useful for hemp breeders. Five main chemotypes (I through V) are recognized and/or proposed today (Small and Beckstead 1973; Mandolino and Carboni 2004); the mode of inheritance of the first three (I: prevalent cannabidiol, CBD; III: prevalent tetrahydrocannabinol, THC; and II: mixed CBD and THC), has been recently extensively studied and clarified (de Meijer et al. 2003; Mandolino et al. 2003; Mandolino 2004). CBD prevalence in the plant (as observed, for example, in most modern fibre cvs.) is determined by homozygosity at a single locus, *B*, of the allele B_D, while prevalence of THC in the plant (e.g. in the drug varieties) is largely determined by homozygosity at the same locus for the B_T allele. Therefore, the chemotype is simply determined by the allelic constitution at one single locus, while the total amount of cannabinoids can be considered a quantitative trait; the combination of these two determination types, yields the specific cannabinoid's profile of a genotype (Mandolino 2004). The other two chemotypes proposed (IV: prevalent CBG, and V: zero cannabinoids) are still poorly characterized, though recently genetical models have been proposed also for chemotype IV (de Meijer and Hammond 2005). Markers associated to the allelic status at the *B* locus (B_D/B_D, B_D/B_T or B_T/B_T) have been developed by bulk segregant analysis of segregating F2 progenies (de Meijer et al. 2003; Mandolino et al. 2003).

The genes responsible for the synthesis of the two main cannabinoids, the non-psychoactive CBD and the psychoactive THC, have been characterized, and several sequences related with the enzyme activities involved are deposited in the Gene Bank (Sirikantaramas et al. 2004). The availability of the gene sequences involved in the synthesis of the two main cannabinoids, and the knowledge of their characteristics, has also led to the development of highly predictive sequence-based PCR markers for the chemotype (Kojoma et al. 2005); besides, marker systems based on multiplex

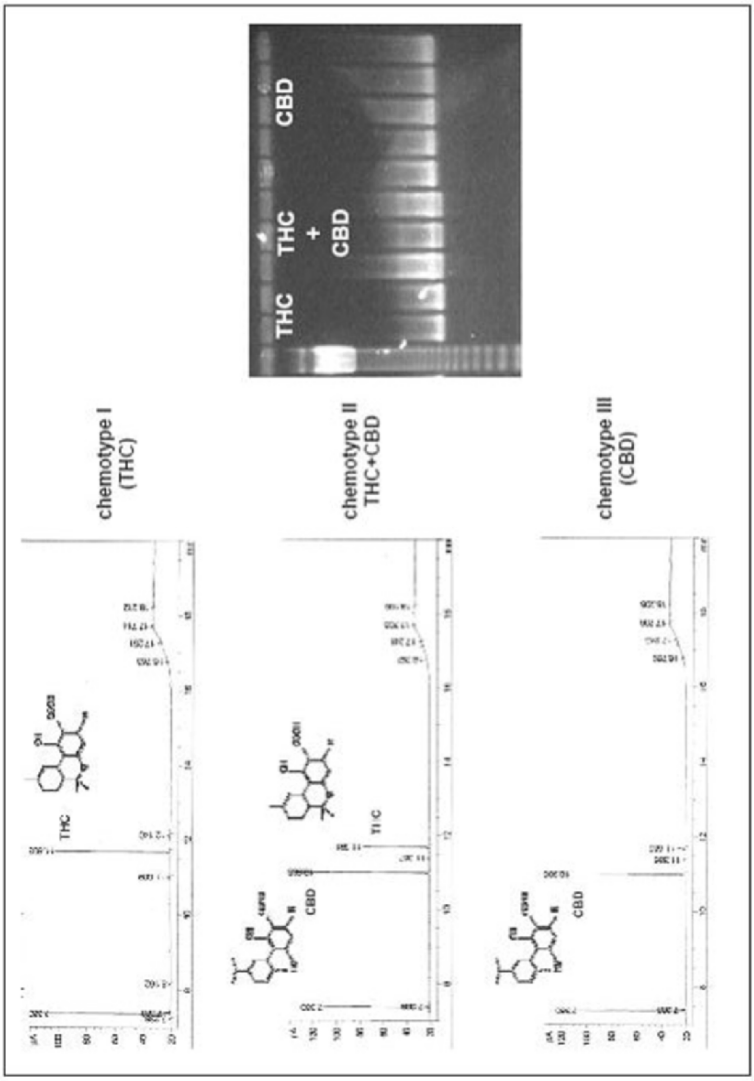


Fig. 3.2. The marker-assisted selection for chemotype in hemp.

PCR amplification, capable of identifying both alleles (and therefore either homozygous or heterozygous genotypes at the *B* locus) have been developed; this particularly effective marker system has been extensively used in the marker-assisted elimination of THC-containing plants (genotyped B_T/B_T) from newly-developed hemp varieties. As expected, these markers, very much like the male-specific markers described above, have a 100% efficiency of correct identification of the genotype at the *B* locus, and also gives indirect evaluation of the absolute THC content of any Cannabis plant (Pacifico et al. 2006). Besides, the same marker can be exploited for selecting high-THC producing plants, today used as a source of this important active principle by pharmaceutical industry (Guy and Stott 2004).

Traditionally, the main product of industrial hemp cultivation was its fibre; the genetics and functional genetics of fibre production is still in its infancy, not only in hemp, but also in other fibre crops. It is likely that the study of fibre characteristics and of their development will play a major role in marker assisted selection for this trait; the functional genomics approach is at its beginning, but is already generating a number of interesting results, both from the point of view of the basic research and of the development of diagnostic tools (see chapter 6 of this book for a review of fibre genomics).

5. Conclusions

In this review, the main advancements achieved in marker-assisted selection and genomics of three industrial crops have been highlighted. These three crops represent somehow three different stages of maturity of the markers' exploitation for genetic improvement. Rapeseed has a well defined and consolidated body of knowledges about the structure and organization of its genome and the several genes relevant from the point of view of industrial utilization, and it can exploit the knowledge of the complete sequence of *Arabidopsis thaliana*; sugar beet is less characterized, especially from the functional genomics point of view, but recent research efforts, and the fact that sugar beet is becoming a model species for root crops, are rapidly filling the gap; hemp genomics still is in its infancy, but a number of important traits, such as sex and chemotype are already characterized, and effective tools for marker assisted breeding have already been developed. It can be foreseen that in the next few years, pushed by

the recent new opportunities of industrial utilization of these crops, important breakthrough in genomics and genetic mapping of other important traits will occur.

References

Alghanim HJ & Almirall JR (2003) Development of microsatellite markers in *Cannabis sativa* for DNA typing and genetic relatedness analysis. Anal Bioanal Chem 376: 1225-1233

Asher MJC (1993). Rhizomania. In: Cooke DA and Scott RK (eds) The sugar beet crop. Chapman and Hall, London, pp 311-346

Barth C and Jander G (2006) Arabidopsis myrosinases TGG1 and TGG2 have redundant function in glucosinolate breakdown and insect defence. Plant J 46: 549-562

Bartsch D, Stevanato P, Lehnen M, Mainolfi A, Mücher T, Moschella A, Driessen S, Mandolino G, Hoffmann A, De Biaggi M, Wehres U, Saeglitz C and Biancardi E (2002) Biodiversity of sea beet in Northern Italy. In: Proc. of the 65th IIRB Congress, Brussels, pp 171-180.

Barzen E, Mechelke W, Ritter E, Seitzer JF and Salamini F (1992) RFLP markers for sugar beet breeding: chromosomal linkage maps and location of major genes for rhizomania resistance, monogermy and hypocotyl colour. Plant J 2: 601-611

Barzen E, Stahl R, Fuchs E, Borchardt DC and Salamini F (1997) Development of coupling-repulsion phase SCAR markers diagnostic for the sugar beet *Rr1* allele conferring resistance to rhizomania. Mol Breeding 3: 231-238

Biancardi E, Lewellen RT, De Biaggi M, Erichsen AW and Stevanato P (2002). The origin of rhizomania resistance in sugar beet. Euphytica 127: 383-397

Botstein D, White RL, Skolnik M and Davis RW (1980) Construction of a genetic linkage map in man using restriction fragment length polymorphism. Am J Hum Genet 32: 314-331

Brunel D, Froger N and Pelletier G (1999) Development of amplified consensus genetic markers (ACGM) in *Brassica napus* from *Arabidopsis thaliana* sequences of known biological function. Genome 42: 387- 402

Cai D, Kleine M, Kifle S, Harloff HJ, Sandal NN, Marcker KA, Klein-Lankhorst RM, Salentijn EMJ, Lange W, Stiekema WJ, Wyss U, Grundler FMW and Jung C (1997) Positional cloning of a gene for nematode resistance in sugar beet. Science 275: 832-834

Carboni A, Paoletti C, Moliterni VMC, Ranalli P and Mandolino G (2000) Molecular markers as genetic tools for hemp characterization. In: Proc. Bioresource Hemp, Wolfsburg

Cheung WY and Landry BS (1996) Current status of genome mapping in the Cruciferae. In: Paterson AH (ed) Genome mapping in plant. Academic Press, 193-210

Coyle HM, Shutler G, Abrams S, Hanniman J, Neylon S, Ladd C, Palmbach T and Lee HC (2003) A simple DNA extraction method for marijuana samples used in amplified fragment length polymorphism (AFLP) analysis. J Forensic Sci 48: 343-347

Cureton AN, Burns MJ, Ford-Lloyd BV and Newbury HJ (2002) Development of simple sequence repeat (SSR) markers for the assessment of gene flow between sea beet (*Beta vulgaris* ssp. *maritima*) populations. Mol Ecology Notes 2: 402-403

de Meijer EPM, Bagatta M, Carboni A, Crucitti P, Moliterni VMC, Ranalli P and Mandolino G (2003) The inheritance of chemical phenotype in *Cannabis sativa* L. Genetics 163: 335-346

de Meijer EPM and Hammond KM (2005) The inheritance of chemical phenotype in *Cannabis sativa* L. (II): Cannabigerol predominant plants. Euphytica 145: 189-198

Desplanque B, Boudry P, Broomberg K, Samitou-Laprade P, Cuguen J and van Dijk H (1999) Genetic diversity and gene flow between wild, cultivated and weedy forms of *Beta vulgaris* L. (Chenopodiaceae), assessed by RFLP and microsatellite markers. Theor Appl Genet 98: 1194-1201

Excoffier L, Smouse PE and Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131: 479-491

Faeti V, Mandolino G and Ranalli P (1996) Genetic diversity of *Cannabis sativa* germplasm based on RAPD markers. Plant Breeding 115: 367-370

Flachowsky H, Schumann E, Weber WE and Peil A (2001) Application of AFLP for the detection of sex-specific markers in hemp. Plant Breeding 120: 305-309

Forapani S, Carboni A, Paoletti C, Moliterni VMC, Ranalli P and Mandolino G (2001) Comparison of hemp (*Cannabis sativa* L.) varieties using Random Amplified Polymorphic DNA markers. Crop Sci 41: 1682-1689

Gao D, Guo D and Jung C. (2001) Monosomic addition lines of Beta corolliflora Zoss in sugar beet: cytological and molecular-marker analysis. Theor Appl Genet 103: 240-247

Gao M, Li G, Yang B, McCombie WR and Quiros CF (2004) Comparative analysis of a Brassica BAC clone containing several major aliphatic glucosinolate genes with its corresponding Arabidopsis sequence. Genome 47: 666-679

Gillan K, Cole MD, Linacre A, Thorpe JW and Watson ND (1995) Comparison of *Cannabis sativa* by Random Amplified Amplified of Polymorphic DNA (RAPD) and HPLC of cannabinoids: a preliminary study. Sci Justice 35: 169-177

Gilmore S and Peakall R (2003) Isolation of microsatellite markers in *Cannabis sativa* L. (marijuana). Mol Ecol Notes 3: 105-108

Gilmore S, Peakall R and Robertson J (2003) Short Tandem Repeat (STR) DNA markers are hypervariable and informative in *Cannabis sativa*: implications for forensic investigations. Forensic Sci Intl 131: 65-74

Guy GW and Stott CG (2004) Cannabinoids for the pharmaceutical industry. Euphytica 140: 83-93

Hagihara E, Itchoda N, Habu Y, Iida S, Mikami T and Kubo T (2005) Molecular mapping of a fertility restorer gene for Owen cytoplasmic male sterility in sugar beet. Theor Appl Genet 111: 250-255

Hasan M, Seyis F, Badani AG, Pons-Kühnemann J, Friedt W, Lühs W and Snowdon RJ (2006) Analysis of genetic diversity in the *Brassica napus* L. gene pool using SSR markers. Genet Res Crop Evol 53: 793-802

Heijbroek W, Roelands AJ and de Jong JH (1983) Transfer of resistance to beet cyst nematode from *Beta patellaris* to sugar beet. Euphytica 32: 287-298

Heller R, Schondelmaier J, Steinrücken G and Jung C (1996) Genetic localization of four genes for nematode (*Heterodera schachtii* Schm.) resistance in sugar beet (*Beta vulgaris* L.). Theor Appl Genet 92: 991-997

Hjerdin-Panagopoulos A, Kraft T, Rading IM, Tuvesson S and Nilsson NO (2002) Three QTL regions for restoration of Owen CMS in sugar beet. Crop Sci 42: 540-544

Hsieh HM, Hou RJ, Tsai LC, Wei CS, Liu SW, Huang LH, Kuo YC, Linacre A and Lee JCI (2003) A highly polymorphic STR locus in *Cannabis sativa*. Forensic Sci Intl 131: 53-58

Hunger S, Di Gaspero G, Möhring S, Bellin D, Schäfer-Pregl R, Borchardt DC, Durel CE, Werber M, Weisshaar B, Salamini F and Schneider K (2003) Isolation and linkage analysis of expressed disease-resistance gene analogues of sugar beet (*Beta vulgaris* L.). Genome 46: 70-82

Jagadish V, Robertson J and Gibbs J (1996) A RAPD analysis distinguished *Cannabis sativa* samples from different sources. Forensic Sci Int 79: 113-121

Jung C, Pillen K, Frese L, Fähr S and Melchinger AE (1993) Phylogenetic relationships between cultivated and wild species of the genus Beta revealed by DNA "fingerprinting". Theor Appl Genet 86: 449-457

Karus M and Vogt D (2004) European hemp industry: cultivation, processing and product lines. Euphytica 140: 7-12

Kleine M, Voss H, Cai D and Jung C (1998) Evaluation of nematode-resistant sugar beet (*Beta vulgaris* L.) lines by molecular analysis. Theor Appl Genet 97: 896-904

Klein-Lankhorst RM, Salentijn EMJ, Dirkse WG, Arens-de Reuver M and Stiekema WJ (1994) Construction of a YAC library from a *Beta vulgaris* fragment addition and isolation of a major satellite DNA cluster linked to the beet cyst nematode resistance locus $Hs1^{pat-1}$. Theor Appl Genet 89: 426-434

Kojoma M, Iida O, Makino Y, Sekita S and Satake M (2002) DNA fingerprinting of *Cannabis sativa* using Inter-Simple Sequence Repeat (ISSR) amplification. Planta Medica 68: 60-63

Kojoma M, Seki H, Yoshida S and Muranaka T (2005) DNA polymorphisms in the tetrahydrocannabinolic acid (THCA) synthase gene in "drug-type" and "fiber-type" *Cannabis sativa* L. For Sci Intl, in press.

Lagerkrantz U and Lydiate DJ (1996) Comparative genome mapping in Brassica. Genetics 144: 1903-1910

Lagerkratz U (1998) Comparative mapping between *Arabidopsis thaliana* and *Brassica nigra* indicates that Brassica genomes have evolved through extensive genome replication accompanied by chromosome fusion and frequent rearrangements. Genetics 150: 1217-1228

Landry, BS, Hubert N and Etoh T (1991) A genetic map for *Brassica napus* based on restriction fragment length polymorphisms detected with expressed DNA sequences. Genome 34: 543-552

Lange W, Brandenburg WA and de Bock TSM (1999). Taxonomy and cultonomy of beet (*Beta vulgaris* L.). Bot J Linn Soc 130: 81-96.

Lewontin RC and Hubby JL (1966) A molecular approach to the study of genetic heterozygosity in natural populations. II. Amount of variation and degree of heterozygosity in natural populations of *Drosophila pseudoobscura*. Genetics 54: 577-594

Li G and Quiros CF (2001) Sequence-related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: its application to mapping and gene tagging in Brassica. Theor Appl. Genet 103: 455-461

Luterbacher MC, Asher MJC, Beyer W, Mandolino G, Scholten OE, Biancardi E, Mechelke W, Slyvchenko O, and Frese L (2005) Sources of resistance to diseases of sugar beet in related Beta germplasm: II. Soil-borne diseases. Euphytica 141: 49-63

Lydiate D, Sharpe A, Lagerkrantz U and Parkin I (1993) Mapping the Brassica genome. Outlook Agric 2. 85-92

Lydiate D and Sharpe A (2003) Aligning genetic maps of *Brassica napus* using microsatellite markers. In: Proc. of the PAG-XI, Plant and Animal Genomes Conference, San Diego, p 473.

Mahmood T, Ekuere U, yeh F, Good AG and Stringam GR (2003) Molecular mapping of seed aliphatic glucosinolates in *Brassica juncea*. Genome 46: 753-760

Mandolino G (2004) Again on the nature of inheritance of chemotype. Letter to the editor. J Ind Hemp 9: 5-7

Mandolino G, Carboni A, Forapani S, Faeti V and Ranalli P (1999) Identification of DNA markers linked to the male sex in dioecious hemp (*Cannabis sativa* L.). Theor Appl Genet 98: 86-92

Mandolino G, Carboni A, Bagatta M, Moliterni VMC and Ranalli P (2002) Occurrence and frequency of putatively Y chromosome linked DNA markers in *Cannabis sativa* L. Euphytica 126: 211-218

Mandolino G and Ranalli P (2002) The application of molecular markers in genetics and breeding of hemp. J Ind Hemp 7: 7-23

Mandolino G, Bagatta M, Carboni A, Ranalli P and de Meijer E (2003) Qualitative and quantitative aspects of the inheritance of chemical phenotype in Cannabis. J Ind Hemp 8: 51-72

Mandolino G and Carboni A (2004) Potential of marker assisted selection in hemp genetic improvement. Euphytica 140: 107-120

Michelmore RW, Paran I and Kesseli RV (1991). Identification of markers linked to isease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. Proc. Natl. Acad. Sci. USA 88: 9828-9832.

Mitchell SE, Kresovich S, Jester CA, Hernandez CJ and Szewc-McFadden AK (1997) Application of multiplex PCR and fluorescence-based, semi-automated allele sizing technology for genotyping plant genetic resources. Crop Sci 37: 617-624

Möhring S, Salamini F and Schneider K (2004) Multiplexed, linkage group-specific SNP marker sets for rapid genetic mapping and fingerprinting of sugar beet (*Beta vulgaris* L.). Mol Breeding 14: 475- 488

Moliterni VMC, cattivelli L, Ranalli P and Mandolino G (2004) The sexual differentiation of Cannabis sativa L.:a morphological and molecular study. Euphytica 140: 95-106

Nagamine T, Todd GA, McCann KP, Newbury HJ and Ford-Lloyd BV (1989) Use of restriction fragment length polymorphism to fingerprint beets at the genotype and species levels. Theor Appl Genet 78: 847-851

Oléo M, van Geyt JPC, Lange W and de Bock TSM (1986) Investigations on an intespecific hybrid involving three species of the genus Beta, with special reference to isozyme polymorphism. Theor Appl Genet 73: 261-266

Pacifico D, Miselli F, Micheler M, Carboni A, Ranalli P and Mandolino G (2006) Genetics and marker-assisted selection of the chemotype in *Cannabis sativa* L. Mol Breeding 17: 257-268

Parkin IAP, Gulden SM, Sharpe AG, Lukens L, Trick M, Osborn TC and Lydiate DJ (2005) Segmental structure of the *Brassica napus* genome based on comparative analysis with *Arabidopsis thaliana*. Genetics 171: 763-781

Paterson AH, Bowers JE, Burow MD, Draye X, Elsik CG, Jiang CX, Katsar CS, Lan TH, Lin YR, Ming R and Wright RJ (2000) Comparative genomics of plant chromosomes. Plant Cell 12: 1523-1540

Peil A, Flachowsky H, Schumann E and Weber WE (2003) Sex-linked AFLP markers indicate a pseudoautosomal region in hemp (*Cannabis sativa* L.). Theor Appl Genet 107: 102-109

Pillen K, Steinrücken G, Herrmann RG and Jung C (1993) An extended linkage map of sugar beet (*Beta vulgaris* L.) including nine putative lethal genes and the restorer gene X. Plant Breeding 111: 265-272

Pradhan AK, Gupta V, Mukhopadhyay A, Arumugam N, Sodhi YS and Pental D (2003 A high-density linkage map in *Brassica juncea* (Indian mustard) using AFLP and RFLP markers. Theor Appl Genet 106: 607-614

Ranalli P, Di Candilo M, Mandolino G, Grassi G and Carboni A (1999) Hemp for sustainable agricultural systems. Agro-Food-Industry Hi-Tech 2: 33-38

Raybould AF, Mogg RJ and Clarke RT (1996) The genetic structure of *Beta vulgaris* ssp. *maritima* (sea beet) populations: RFLPs and isozymes show different patterns of gene flow. Heredity 77: 245-250

Richards CM, Brownson M, Mitchell SE, Kresovich S and Panella L (2004) Polymorphic microsatellite markers for inferring diversity in wild and domesticated sugar beet (*Beta vulgaris*). Mol Ecology Notes 4: 243-245

Rode J, In-Chol K, Saal B, Flachowski H, Kriese U and Weber WE (2005) Sex-linked SSR markers in hemp. Plant Breeding 124: 167-170

Sakamoto K, Ohmido N, Fukui K, Kamada H and Satoh S (2000) Site-specific accumulation of a LINE-like retrotransposon in a sex chromosome of the dioecious plant *Cannabis sativa*. Plant Mol Biol 44: 723-732

Sakamoto K, Abe T, Matsuyama T, Yoshida S, Ohmido N, Fukui K and Satoh S (2005) RAPD markers encoding retrotransposable elements are linked to the male sex in Cannabis sativa L. Genome 48: 931-936

Salentijn EMJ, Sandal NN, Lange W, de Bock TSM, Krens FA, Marcker KA and Stiekema WJ (1992) Isolation of DNA markers linked to a beet cyst nematode resistance locus in *Beta patellaris* and *Beta procumbens*. Mol Gen Genet 235: 432-440

Schmidt T, Junghans H and Metzlaff M (1990) Construction of *Beta procumbens*-specific DNA probes and their application for the screening of *B. vulgaris* x *B. procumbens* (2n=19) addition lines. Theor Appl Genet 79: 177-181

Schneider K, Borchardt D, Schäfer-Pregl R, Nagl N, Glass C, Jeppson A, Gebhardt C and Salamini F (1999) PCR-based cloning and segregation analysis of functional gene homologues in *Beta vulgaris*. Mol Gen Genet 262: 515-524

Schneider K, Weisshaar B, Borchardt DC and Salamini F (2001) SNP frequency and allelic haplotype structure of *Beta vulgaris* expressed genes. Mol Breeding 8: 63-74

Scholten OE, de Bock TSM, Klein-Lankhorst RM and Lange W (1999) Inheritance of resistance to beet necrotic yellow vein virus in *Beta vulgaris* conferred by a second gene for resistance. Theor Appl Genet 99: 740-746

Schumacher K, Schondelmaier J, Barzen E, Steinrücken G, Borchardt DC, Weber WE, Jung C and Salamini F (1997) Combining different linkage maps in sugar beet (*Beta vulgaris* L.) to make one map. Plant Breeding 116: 23-38

Siniscalco Gigliano G, Caputo P and Cozzolino S (1997) Ribosomal DNA analysis as a tool for the identification of *Cannabis sativa* L. specimens of forensic interest. Sci Justice 37: 171-174

Sirikantaramas S, Morimoto S, Shoyama Y, Ishikawa Y, Wada Y, Shoyama Y and Taura F (2004) The gene controlling marijuana psichoactivity. J Biol Chem 279: 39767-39774

Skaracis GN and Biancardi E (2000) Breeding for Cercospora resistance in sugar beet. Adv Sugar Beet Res 2: 177-196

Small E and Beckstead HD (1973) Common cannabinoid phenotypes in 350 stocks of *Cannabis*. Lloydia 36: 144-165

Somers J, Rakow G and Rimmer R (2002) *Brassica napus* DNA markers linked to white rust resistance in *Brassica juncea*. Theor Appl Genet 104: 1121-1124

Speckmann GJ and de Bock TSM (1982) The production of alien monosomic additions in *Beta vulgaris* as a source for the introgression of resistance to beet root nematode (*Heterodera schachtii*) from Beta species of the Section *Patellares*. Euphytica 31: 313-323

Stevanato P, De Biaggi M, Skaracis GN, Colombo M, Mandolino G, and Biancardi E (2001) The sea beet (*Beta vulgaris* L. ssp. *maritima*) of the Adriatic coast as source of resistance for sugar beet. Sugar Tech 3: 77-82

Sturtevant AH (1913) The linear arrangement of six sex-linked factors in Drosophila, as shown by their mode of association. J Exp Zoology 14: 43-59

Teutonico RA and Osborn TC (1994) Mapping of RFLP and qualitative trait loci in *Brassica rapa* and comparison to the linkage maps of *B. napus*, *B. oleracea*, and *Arabidopsis thaliana*. Theor Appl. Genet 89: 885-894

The Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 408: 796-815

Tufto J, Raybould AF, Hindar K and Engen S (1998) Analysis of genetic structure and dispersal patterns in a population of sea beet. Genetics 149: 1975-1985

UN (1935) Genome analysis in Brassica with special reference to the experimental formation of *B. napus* and its peculiar mode of fertilization. Japan J Bot 7: 389-452

Weber WE, Borchardt DC and Koch G (1999) Combined linkage maps and QTLs in sugar beet (*Beta vulgaris* L.) from different populations. Plant Breeding 118: 193-204

Weber WE, Borchardt DC and Koch G (2000) Marker analysis for quantitative traits in sugar beet. Plant Breeding 119: 97-106

Weiland J and Koch G (2004) Sugarbeet leaf spot disease (*Cercospora beticola* Sacc.). Mol Plant Pathology 5: 157-166

Wilkinson M and Linacre A (2000) The detection and persistence of *Cannabis sativa* DNA on skin. Sci Justice 40: 11-14

Zhao J and Meng J (2003) Genetic analysis of loci associated with partial resistance to *Sclerotinia sclerotiorum* in rapeseed (*Brassica napus*). Theor Appl Genet 106: 759-764

4

Improved agronomy and management of crop plants for industrial end uses

Lorenzo Barbanti, Andrea Monti, Gianpietro Venturi

Department of Agro-environmental Science and Technology (DiSTA), Viale Fanin 44, 40127 Bologna, Italy (e-mail:Lorenzo.barbanti@unibo.it)

1. General concepts

Agronomy, the science ruling the fields, has the privilege and the task of dealing with many aspects and related disciplines, covering the biological and the physical sphere. Harmonizing the complex of interactions arising form the organisms, factors and conditions involved in the process of plant growth is not an easy task, as general. Optimising plant growth and crop production, while safeguarding the environment, often proves a harder challenge. In this light, the crops for industrial end-uses may intrinsically be seen as crops of potential large scale, thus exerting a significant influence - both positive and negative – on the environment.

Crop management in its two aspects of quality and intensity is deeply involved in this influence, although the effects of crop inputs and techniques are seldom consistent, depending on the specific input, its level, the crop to which it is applied, other cropping conditions. The same is true for the long-studied influence of inputs/techniques on crop yield and quality. Yield and quality represent a good example of the problems to face, especially when they are adversely correlated, such as in the case of nitrogen fertilization in nitrogen-sensitive crops (e.g. sugar beet), requiring a compromise between contrasting effects; adding the environmental issue to the already-existing dualism of yield vs. quality, involves a higher-level

P. Ranalli (ed.), Improvement of Crop Plants for Industrial End Uses, 83–127.

compromise to be looked for, within what would otherwise be seen as "good" husbandry.

As to this, a common background linking most industrial crops is that, given the present financial context and the intrinsic nature of their products, they are grown in the effort to optimise the efficiency of all external inputs, such as fertilizers, irrigation, subsidiary energy spent in soil tillage, etc.. The process is susceptible of minimizing the load of chemicals on the environment, which is, in principle, a desirable outcome. On the other hand, the tendency to optimise inputs according to the expected response, disregarding the computation of mass balances, may trigger a progressive depletion of soil reserves, that is evident in the case of nutrients, but may extend to soil moisture, if a crop with a high ability to exploit soil water reserves is followed by another at lower aptitude, in the lack of restoring rainfalls. Referred to nutrients, this occurrence goes under the name of "soil mining" and represents a potential constraint to future production, which seems wiser to prevent than to recover from.

From the above discussion, it is perceived that the guidelines of agronomic improvement in crops for industrial end uses must follow a narrow pathway, in order to comply with opposite aims. In the specific of crop management, the operations that are needed to grow a crop may be grouped under a few basic categories: i) tillage/establishment of the crop, including seeding or transplanting of the breeding material; ii) nutrition, based on chemical and organic fertilization; iii) water management, covering both irrigation and adaptation to drought; iv) protection from competition and biotic stresses, i.e. weeds, pests and diseases; v) harvest/conditioning, which is the subject of closer relationship between agriculture and industry, focusing on harvest campaigns, product storage, supplying fluxes to industrial plants, etc..

Analysing the recent progress and the pending problems in each category, in the present agricultural context and with the available tools, seems the best way to outline a management for crops at industrial end uses.

2. Soil tillage and crop establishment

Soil tillage and crop establishment cover a vast array of interventions, ranging from none, in the case of already-established perennial crops, to a sequence of high intensiveness, e.g. in crops requiring a deep tillage, the preparation of a fine seedbed, and a low-speed seeding or transplanting.

2.1 Soil tillage

The modern approach in soil tillage is aimed at enhancing soil fertility, a task which is deeply embedded in traditional agronomy and does not represent, in itself, anything new.

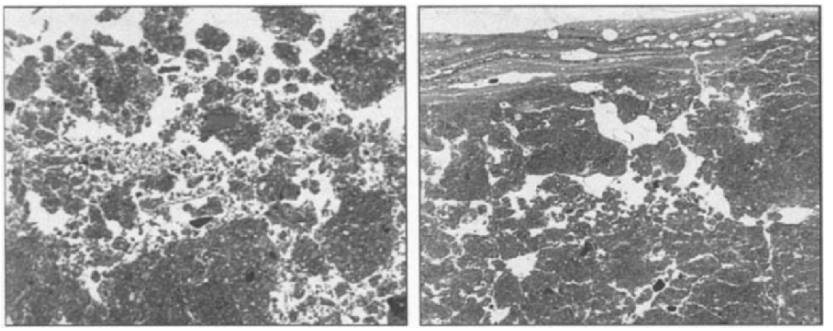


Fig. 4.1. Macro-photographs of vertically oriented thin sections prepared from undisturbed samples from the surface layer (0-100 mm) of soil tilled by continuous deep ploughing (left) and the same soil after raindrop impact (right). Surface crust formation is very evident. Frame length 35 mm x 28 mm. Reprinted from Soil Tillage Research, 79, Pagliai et al. Soil structure and the effect of management practices, 131-143, © 2004 Elsevier B.V., with permission from Elsevier.

Other tasks commonly associated with soil tillage, such as the incorporation of crop residues, fertilizers and weeds, are still performed, but alternatives are increasingly available, such as herbicides, fluid-fertilizer injection, crop-residue mulching, whose adoption deprives soil tillage of part of its former importance. Even within the vast issue of soil fertility, different concepts and purposes are now comprised with respect to the past. Chemical fertility, still a main issue in crop production, is a more appropriate concern of fertilization. Conversely, physical and biological fertility are the core of a modern policy of soil management, since the influence played by soil tillage cannot be easily replaced. Physical and biological fertility lie on the common principle that undisturbed soils are normally characterized by the best status. In agricultural soils, all the efforts are aimed at mimicking nature's action as much as possible, through tillage (Fig. 4.1). There is no single method corresponding to this concept, since the same results may be achieved through different ways. In other words, all the tools (inverting, non-inverting ones) and the techniques (deep, shallow, no tillage) are still valid and applicable, depending on specific aims and conditions.

In recent literature, the vast combination of tillage depths and instruments are investigated, in view of a sustainable crop production. Many parameters are analysed as potential soil-quality indicators. Among them,

soil bulk density is considered a good indicator of soil structure, the high levels indicating compaction by heavy traffic and, to a lesser extent, by re-consolidation of the tilled layer (Botta et al. 2006; Hamilton-Manns et al. 2002).

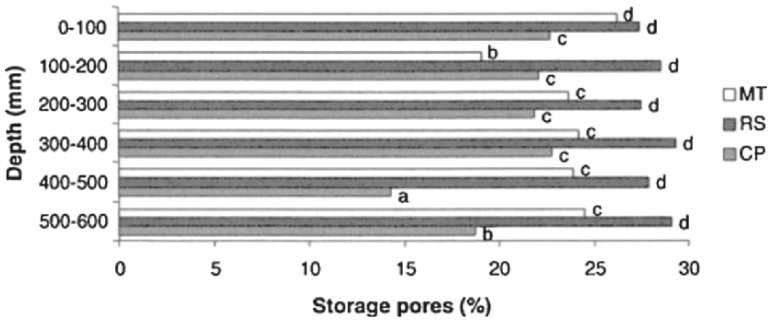


Fig. 4.2. Effect of tillage systems on elongated transmission pore distribution along soil profile expressed as a percentage of total area occupied by pores ranging from 50-500 μm per thin section (MT, minimum tillage; RS, ripper sub-soiling; CP, conventional deep ploughing). Values at each depth with different letters are significantly different at $P \leq 0.05$. Reprinted from Soil Tillage Research, 79, Pagliai et al. Soil structure and the effect of management practices, 131-143, © 2004 Elsevier B.V., with permission from Elsevier.

In this respect, no tillage and sod-seeding of the crop is responsible for higher bulk density in the top 0.08 to 0.15 m, compared to reduced and conventional tillage (Bescansa et al. 2006; Bhattacharyya et al. 2006; Singh and Malhi 2006; Dam et al. 2005; Deen and Kataki 2003). The result is an increase in soil strength, leading to a higher resistance to penetration, whereas water infiltration appears both positively (Liebig et al. 2004) and negatively (Singh and Malhi 2006) influenced by no-till. These effects generally end or reverse at deeper layers (> 0.15 m). In terms of bulk density and porosity, non-inverting, reduced tillage generally has an intermediate behaviour between ploughing and no tillage: in the case of ripper sub-soiling, a higher macro-porosity associated with a homogeneous distribution along soil profile has been observed (Pagliai et al. 2004), due to a larger number of elongated transmission pores, easing the passage of water through the profile (Fig. 4.2). The stability of soil aggregates to water is, consequently, improved, mitigating the tendency to soil crusting, an effect shown also for no tillage (Liebig et al. 2004). The resistance to wind erosion is improved by reduced and no tillage, too, thanks to larger aggregates, less sensitive to wind action (Malhi et al. 2006; Singh and Malhi 2006). So it is perceived that a reduced tillage intensity is possible or even beneficial to soil physical state and should, therefore, be economically pursued also in industrial crops. Examples are given for oilseed rape, which

has achieved satisfactory yields in a review of the studies run in the Scandinavian countries on reduced tillage and direct drilling (Rasmussen 1999).

Table 4.1. Soil organic carbon sequestration rates (0-0.2 m soil depth) upon conversion from conventional tillage to no-till. Adapted from Agriculture, Ecosystems and Environment, 111, Tan and Lal, Carbon sequestration potential estimates with changes in land use and tillage practice in Ohio, USA, 140-152, © 2005 Elsevier B.V., with permission from Elsevier.

Location [a]	Duration year	Antecedent C content (g C m^{-2})	C change rate (g C m^{-2} yr^{-1})	
			Mean	Std dev.
Choshocton	17	924	47	9
Hoytville	16-19	589	50	36
Wooster	18-30	396	60	9
S. Charleston	18-28	266	87	50
Mean			62	29

[a] Several data sources and different soil taxa.

In sweet and fibre sorghum, the combined effect of shallow tillage (0.15 m) and low N and P fertilization (60 and 35 kg ha^{-1}, respectively), compared to conventional ploughing (0.3 m) and normal N and P rates (120 and 70 kg ha^{-1}, respectively), entailed a certain loss in biomass yield only in one year out of three (Amaducci et al. 2004), but the effect is not clearly attributable to soil tillage alone. Sunflower is another industrial crop investigated under different tillage, but the effects observed are more related to soil moisture as influenced by soil tillage, and later discussed, in the section on water management. In this crop, anyway, reduced tillage (chisel at 0.25-0.3 m) has been observed to limit early plant growth and N-uptake with respect to traditional one (ploughing), but not final seed yield and quality (Murillo et al. 1998); i.e. the more-compact crop maintained its yield potential, under relatively-dry conditions (Southern Spain).

The level of soil organic matter and the carbon-sink effect of soils are other important issues that may benefit from reduced tillage. No-tillage entails a lower mineralization of native organic matter and may contribute to a net CO_2 sequestration (Tan and Lal 2005) (Table 4.1), sometimes only in association with other factors such as stubble retention at the surface (Wang and Dalal 2005). The light fractions of organic matter and of N seem to be more involved in these increases, than the total amounts (Malhi et al. 2006). Also microbial-biomass and potentially mineralizable N, valuable indicators of soil quality, show higher levels in association with no tillage (Liebig et al. 2004; Salinas-García et al. 2002), at least in the surface layer: in fact, a soil-carbon stratification is clearly detectable in

noninverting tillage compared to ploughing, depending on the stronger C sequestration operated by the former in the topsoil (Piovanelli et al. 2005). In this respect, minimum tillage may be seen as a good compromise between no-tillage and ploughing, in terms of organic matter content and distribution along the profile (Duiker and Beegle 2005; Deen and Kataki 2003). Even with the most conservative tillage systems, the balance in organic C may be negative with respect to former semi-natural systems. This is the case of an abandoned 15-year old grassland (Olson et al. 2005), where the loss in C after 12 years of cropping, still modest in no-till (10%), rose with chisel tillage (16%) and was further enhanced by mouldboard ploughing (27%). Seen from a different point of view, the difference of conservative tillage over conventional management represents a positive gain: in the cited research, the annual build-up of the C stock in the root zone (0-0.75 m depth) was set at 0.71 Mg ha^{-1} yr^{-1} for no tillage over ploughing, and at 0.46 for chisel tillage. In agreement, a simulation study showed an increase in the rate of soil carbon sequestration in the range of 0.4-0.8 Mg ha^{-1} yr^{-1}, depending on cropping system, with no-till management compared to conventional tillage (Thomson et al. 2006). At last, an experience on a single ploughing of a soil previously subjected to non-inverting tillage for 7-9 years (Koch and Stockfisch 2006), in order to overcome constraints of compaction, weed and slug infestation, resulted in a loss of organic matter of 6%, less severe than in Olson's et al. (2005) research, thanks to a less-conservative soil management maintained for a shorter time, but still showing the volatility of the benefits capitalized in years of conservation tillage, with the return of ploughing.

Worldwide, the steeply intensifying of agricultural practices in response to policy changes and fast population growth has resulted in an overall declines in soil C. For example, in temperate-zone agriculture, soil C was shown to decrease by 50% during the first 25 years of cultivation under intensive agricultural techniques (Matson et al. 1997). In developing countries, practice is to increase soil tillage, while reducing organic fertilizers and removing crop residues, thus resulting in lower levels of soil C and nutrients, thereby increasing fertilizer needs. For example, Metherell et al. (1995) demonstrated that soil C losses can be up to 50% lower for no-till compared to conventional tillage in a winter wheat-fallow rotation. Thus, while yield per unit area has steadily increased, yield per unit of fertilizer added has declined (Gale et al. 2002). China represents an emblematic example of this trend: the annual average cultivation-induced C losses have been estimated at 15 Mg (C) ha^{-1}, representing a total C-loss of 2 Pg C (Song et al. 2005). The IPCC (Intergovernmental Panel on Climate Change) Second Assessment Report has estimated that over the next 100 years it may be possible to restore two-third of carbon emission through

sustainable agricultural practices such as reduced soil tillage and erosion control.

Table 4.2. Cropping system effect on soil organic carbon (SOC) and total nitrogen (TN) content, soil C:N ratio, and bulk density (BD) in the 0-0.15 and 0.15-0.30 m soil layer, after 10-year implementation of the cropping system experiment under no-tillage management. Adapted from Agriculture, Ecosystems and Environment, 105, Al-Kaisi et al. Soil carbon and nitrogen changes as influenced by tillage and cropping systems in some Iowa soils, 635-647, © 2005 Elsevier B.V., with permission from Elsevier.

Cropping system	SOC (Mg ha^{-1})		TN (Mg ha^{-1})		C:N ratio		BD (g cm^{-3})	
	0-15	15-30	0-15	15-30	0-15	15-30	0-15	15-30
Smooth bromegrass	47,1 a	33,2 a	4,7 a	2,0 a	10,0 b	16,6 b	1,08 b	1,12 c
Switchgrass	40,7 a	26,3 a	2,4 b	0,8 b	17,0 a	32,9 a	1,25 a	1,17 b
Corn-soy-bean-alfalfa	26,7 b	17,0 b	2,1 b	0,8 b	12,7 b	21,3 b	1,27 a	1,29 a

Values in column with different letters are significantly different at $P \leq 0.05$.

It appears, then, as perennial crops have a competitive edge over annual ones in terms of carbon sequestration, and are to be preferred for this aspect, wherever a choice is possible within the same production chain, such as in the case of biomass crops for energy: switchgrass (*Panicum virgatum* L.) for instance, is a perennial grass of potential energy use, that was seen able to raise the soil-carbon content by an average 1.2 Mg ha^{-1} yr^{-1} over 10 years, compared to a typical rotation of temperate regions such as maize, soybean, alfalfa (Al-Kaisi et al. 2005) (Table 4.2).

Again, a five-year old switchgrass was shown to accumulate about 8 Mg ha^{-1} of dry roots, that is about five-times more than maize (Parrish et al. 1997). In the same crop, an increase of 2 g kg^{-1} of soil carbon was observed in just three years of cropping (Sanderson et al. 1997a), and, at five years from planting, about 25% of soil organic carbon originates from the crop (Garten and Wüllschleger 2000). Moreover, compared to traditional crops such as wheat and maize, switchgrass has been found to store the largest amount of C in a relatively-deep layer (0.3-0.9 m), less exposed to mineralization losses (Liebig et al. 2005; Frank et al. 2004).

From the discussed literature, it may be concluded that also industrial crops, despite their heterogeneity, should be able to play a positive role in the context of sustainability, by adopting methods of conservative tillage which, in turn, contribute to mitigate greenhouse gases emissions, to

reduce soil erosion, and to improve the overall efficiency of the energy spent in cropping.

2.2 Crop planting

Crop planting is clearly linked to soil tillage. In the cropping systems at reduced or no-tillage, dedicated seeders have been developed, that are fitted with sturdier coulters or disks, in order to cut through superficial mulches (straw, stalks, desiccated weeds and cover crops) and place the seed at the right depth while improving soil-seed contact. The development of such machines is still in progress, focusing on specific parameters of seedbed quality, as it can be perceived from literature: in a recent research (Vamerali et al. 2006), for instance, a new kind of wide-sweep furrow opener compared with a traditional double-disc opener led to higher soil-residue mixing and lower bulk density in the top 0.05 m. These favourable conditions were, conversely, associated with a certain delay in maize emergence, which might be due to a lower soil/seed contact.

Rolling the seedbed to improve its firmness and the contact between soil and seed is often done in tillage systems, especially for small-seed crops. In one such industrial crop (switchgrass), rolling the seedbed prior to sowing, and in case also after sowing, has been shown to actually improve seedling emergence from 56% to an average 70% in a specific trial (Monti et al. 2001). This may lead to the false belief that a hard soil offers, by definition, favourable emergence conditions. The fact is proven wrong also in the cited experience, where the increase in soil resistance in the top 0.2 m, associated to no tillage, has been responsible for a substantial curb in emergence (Fig. 4.3), especially in the interval between 1 and 2 MPa. It appears, then, as a border should be erected between seedbed compression, a potentially-useful practice, and soil compaction, an unfavourable occurrence from all viewpoints.

The respect of proper seeding conditions also in simplified planting systems, as a premise to good crop stand and early growth, has been investigated in a trial where four different drill configuration were tried in no tillage on maize, wheat and soybean (Chen et al. 2004): removing the press wheel reduced the speed of emergence and the final population on normal and dry field conditions; removing also the gauge wheel resulted in double seeding depth on soft soil in laboratory conditions, leading to the same effects. Although a reduced and delayed emergence seldom affected yields in these trials, the importance of a complete, simultaneous establishment is implicitly supported.

As for transplanted crops, the preparation of the breeding material is a burden often puzzling farmers, that are accustomed to the simplicity of true seeds, or to well-organized supply chains, delivering them the seedlings at the right time and in the right shape (e.g. paper-pots) for their transplanters.

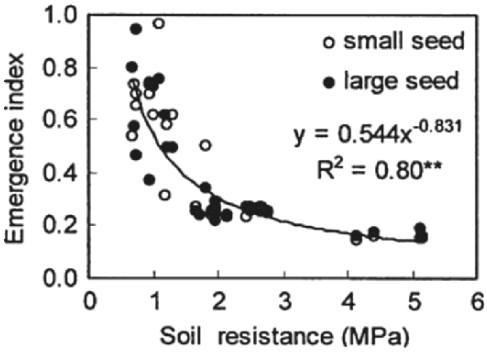


Fig. 4.3. Correlation between soil resistance at a 0-0.2 m depth and emergence index, the ratio of actual to potential seedlings, in small- and large-seed varieties of switchgrass. Reproduced from Soil & Tillage Research, 63, Monti et al. Evaluation of the establishment of lowland and upland switchgrass (*Panicum virgatum* L.) varieties under different tillage and seedbed conditions in northern Italy, 75-83, © 2001 Elsevier B.V., with permission from Elsevier.

For those crops that are normally planted through rhizomes, the preparation of a suitable number involves considerable amounts of time and labour and may, therefore, play a discriminating role against them. Other ways are, therefore, tried: in giant reed (*Arundo donax* L.), one such crop now susceptible of expansion as a biomass for energy in the frame of the Kyoto Protocol, the rush for of an adequate amount of breeding material is pressing towards simpler means than fractured rhizomes, such as stem cuttings placed into the soil with the generation of new plants from axillary buds (Gherbin et al. 2005), and is encouraging also micro-propagation trials.

2.3 Soil management during crop growth

In exchange for the potential complexity in the phases of soil preparation and crop planting, a few cares are generally needed along the cycle in modern industrial crops: over-seeding followed by thinning in the early stages is practically disappearing in the western world even in vegetables such as tomato. Inter-row hoeing is feasible in the crops having an

interrow space wide enough (at least 0.4-0.5 m) to allow it. Its contribute to the control of weeds and, to a lesser extent, of soil evaporation is not negligible, especially in low-input cropping systems, where lower rates of herbicides, if any, are applicable. At last, earthing-up is limited to those crops taking advantage of the extra cover of soil on top of expanding tubers and rhizomes, or from the ridging of soil surface in view of furrow irrigation.

3. Crop nutrition

Crop nutrition is the base, along with light interception, of plant growth and crop production. The various micro- and macro-nutrients play different roles in the plant from a physiological point of view, that seem needless to discuss here. The crucial point is that the plant must be granted a balanced, satisfactory supply of all nutrients, in order to attain the desired growth, under non-limiting conditions of different nature (e.g. drought stress, pests and diseases, etc.). Assuring this condition while respecting other constraints, namely financial and environmental ones, is not an easy task. Mineral and organic fertilization are the tools to achieve the goal, but several other techniques must be considered, that interact with plant nutrition, both in a positive and in a negative way.

Industrial crops intrinsically have variable nutritional needs and responses to applied nutrients, depending on the species and on their destinations. So, a common picture of their behaviour may not be drawn, but a discussion of the concepts now prevailing in plant nutrition may help to better highlight modern trends in their fertilization.

3.1 Nitrogen

Nitrogen is the nutrient of greatest importance and of most delicate management throughout all crops. Apart from the leguminous species, that are little represented among industrial crops, plants mainly take up the nutrient from the pool of soil mineral N, which in turn is generally inadequate to satisfy their requirements, and must be fed by other sources, namely mineral and organic fertilizers. The problem is that nitrogen has a high mobility along soil profile, and may be leached in drainage water or volatilised to the atmosphere. More to that, the biological part of its cycle is not less complicate, since the relationships with soil biomass and specific bacteria

are functional to the cycles of mineralization/immobilization that rule its availability.

Many decision-support systems are currently in use, in order to avoid the risk of nitrogen imbalances. The computation of nutrient budgets (balances), defined as the summary tables of nutrient inputs and outputs (Oenema 2003), may be considered one of the simplest: nutrient budgets may be set at three basic scales: soil system, soil surface (field) and farm gate. The system budget is, of course, specific of trial sites, although its results may be inferred into normal cropping situations, contributing data for useful considerations (Barbanti et al. 2006). The other two may be calculated with relative ease also for commercial crops/farms, although they are only approximate in the assessment of system imbalances: in fact, the application of the soil surface budget for nine years to a rotation including oilseed rape showed that only 13-25% of the surpluses originating from the computation were actually leached (Sieling and Kage 2006), indicating a poor correlation between anticipated and actual amounts.

More sophisticated systems are needed, in order to get more reliable outputs. To this aim, a more advanced approach is to implement actual soil nutrient status into the nutrient budget, as a specific reference. Many systems have been developed and validated, based on soil analysis; among them, one of the most diffused is the balance sheet, first developed in France (Rémy and Hébert 1977; Rémy and Viaux 1982). The principle is to take a representative soil sample at a significant depth (generally 0-0.9 m) and to determine mineral N, at the beginning or in early phases of crop cycle. Then other parameters are assumed, such as the expected mineralization during the cropping season, according to the soil type; the net mineralization/immobilization of organic fertilizers and of preceding crops' residues; the nutrient requirements at a standard (or personalized) yield potential, plus a small amount representing soil residual N-min at harvest. The algebraic sum of these items gives the amount of N to be applied as fertilizer. The method has been successfully in use for large-surface crops, primarily winter wheat and sugar beet. It has further evolved into a software (Machet et al. 1990), and is now implemented into a dynamic soil-crop model for winter wheat (Jeuffroy and Recous 1999) (Fig. 4.4), where it represents the soil sub-model, while the crop sub-model simulates growth and N-uptake according to the crop's simulated radiation use efficiency and to a critical dilution curve for nitrogen.

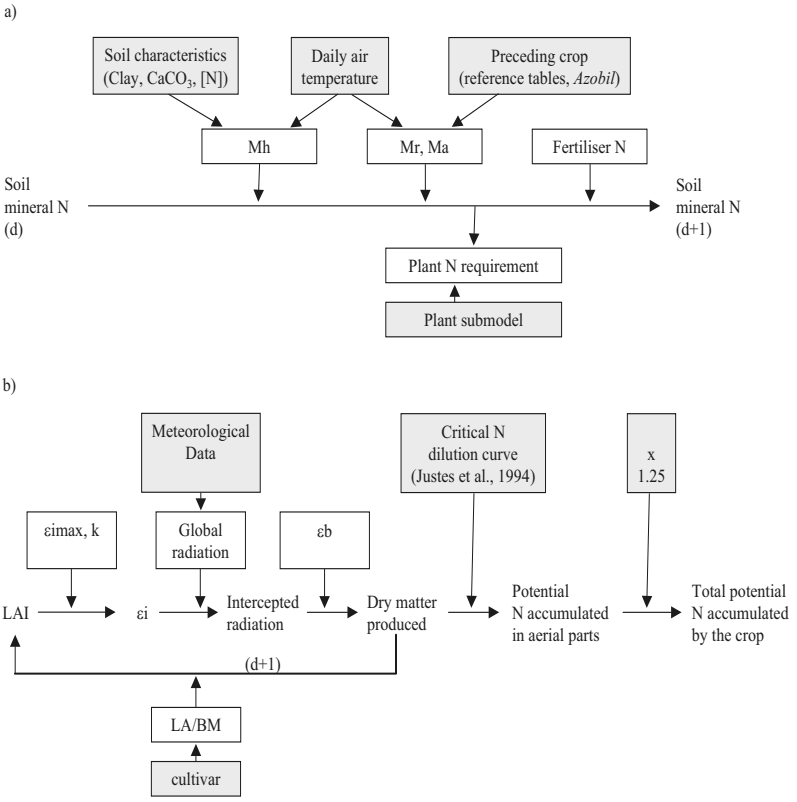


Fig. 4.4. Flow charts of the simulation model of daily soil nitrogen availability (a) and daily plant nitrogen requirements (b). Mh=net mineralization of humus; Mr=net mineralization of crop residues; Ma=net mineralization of organic wastes; εi=fraction of incident radiation intercepted by the crop; εb=radiation use efficiency; εimax and k=maximum εi and extinction coefficient; LA/BM=ratio of leaf area over total aerial biomass; boxes in grey=submodel input. Reproduced from European Journal of Agronomy, 10, Jeuffroy and Recous, Azodyn: a simple model simulating the date of nitrogen deficiency for decision support in wheat fertilization, 129-144, © 1999 Elsevier B.V., with permission from Elsevier.

Other methods have been developed, based on soil analysis of available forms of nitrogen with chemical (e.g., KCl, $CaCl_2$) and electro-chemical (EUF) extraction (Németh 1982; Wiklicky 1982), where the soil nutrient status is the base for the computation of fertilizer rates. None of them has been extended to industrial crops, although the adaptation of existing soil models should not be complicate, possibly requiring only the implementation of specific nitrogen uptakes, crop cycle and rooting depth.

Another large category of tools in nitrogen advising is that of plant indicators, instead of soil ones. Many chemical and optical assessments have been proposed: among them, the lab or field analysis of sap nitrate content in wheat stems and in sugar beet petioles; the rapid chlorophyll readings by means of dedicated tools (e.g. Minolta SPAD 502) based on optical absorbance (Yadava 1986); and the possibility of using ground-based measurements of spectral reflectance (Graeff and Claupein 2003).

Table 4.3. Summary of the main advantages and disadvantages of different sensing platforms. Adapted from Biosystems Engineering, 90, Scotford and Miller, Applications of spectral reflectance techniques in Northern European cereal production: a review, 235-250, © 2005 Elsevier B.V., with permission from Elsevier.

	Space	Aerial	Ground
Area covered per scan	Area scanned increases with platform height		
	typically km^2	typically m^2	typically cm^2
Spatial resolution	Resolution coarseness increases with platform height		
(pixel size)	1-30 m^2	0,05-2 m^2	mm^2 to cm^2
Temporal resolution	Weeks	days	hours
Affect of cloud cover	Influence of cloud increases with platform height		
	heavy influence	moderate infl.	not affected
Affect of local illumination conditions	Influence of illumin. conditions decreases with height		
	not affected	moderate infl.	heavy influence
Availability of data to end user	long delays	some delays	no delays
Control of end user	limited control	some control	full control

These systems are based on the principle of anticipating deficiencies through monitoring, in order to prevent them by fertilizing. They are very useful in horticultural crops, where drip irrigation allows a just-in-time delivery of fertilizers ("fertigation"); still of interest in the winter fertilization of wheat, but are unlikely to extend to crops whose management intrinsically requires simplification, such as many industrial crops.

More to that, their appeal in large-surface crops is rivalled by that of remote sensing, a term including both aerial (plane, helicopter) and space (satellite) sensing, based on spectral-reflectance analysis of crop images, although in a recent experiment (Reyniers et al. 2006) ground-based reflectance (CropScan multi-spectral radiometer) has proved more precise than aerial one (colour infra-red aerial image) in assessing wheat nutritional status and yield components at harvest. In a review (Scotford and Miller 2005), the advantages and disadvantages of the three sensing platforms, ground-, aerial- and space-based, are discussed and summarized (Table 4.3). It is concluded that, in order to provide quantitative crop information to aid input decisions, ground or aerial sensing are more appropriate than satellite one, because of higher spatial and temporal resolution. In another experience

(Jia et al. 2004), aerial true-colour photography has provided normalized colour intensities (red, green and blue bands) that were highly correlated with total N concentration, SPAD readings and sap nitrate in winter wheat, thus showing the possibility of replacing more-expensive terrestrial tools of nutrient assessment. Aerial sensing may, therefore, be seen as a good compromise between efficiency and accuracy, while satellite sensing is a field of promising development, given the commitment of private companies and public boards (e.g. the European Space Agency), but whose interest is presently restricted to simple applications of limited optical resolution, such as targeting crop inspections according to specific agricultural rules.

3.2 Other nutrients

The rest of macro-nutrients and all the micro-nutrients are of minor concern, compared to nitrogen. Yet, they deserve attention, since deficiencies are likely to impair plant growth and, consequently, affect crop yield.

As for the two other relevant macro-nutrients, P and K, sufficiency thresholds have been defined for common soil-crop combinations, using specific extractors. The management of P and K in fertilization has not substantially evolved recently: the two nutrients are either little mobile (K) or substantially static (P) in soil profile, so they need to be incorporated, if satisfactory concentrations are to be found at some depth from the surface. In this light, minimum and no tillage lead to a nutrient stratification, which can be partly overcome by application techniques such as soil injection or placement below the seed. Both recently proved an effective way of application in maize on K-deficient soils after twelve years of no tillage (Vyn et al. 2002). It is also argued that nutrient diagnostic methods should be adjusted to soil management, i.e. soil sampling should be maintained at a certain depth even in no-tillage, in order to have a good correlation between soil P status and wheat response to fertilizer (Zamuner et al. 2005), although another source (Duiker and Beegle 2005) suggests that samples in no tillage could potentially be taken at a shallower depth, provided that calibration curves are available. So there is no general agreement on the matter at present, probably because a bit too few experiences have been done so far. All the discussed concepts concerning P and K fertilization, described for cereals, are likely to apply also to industrial crops, which have been little investigated on this subject.

Likewise, also for secondary and micro-nutrients there is a scarcity of information on industrial crops. As a general, it is expected that industrial crops consist of vigorous plants, little affected by micro-deficiencies and other like constraints, but in fact this may not be stated for sure. The lack of literature supports this view, but the situation might change, especially for crops that are at the beginning of their diffusion. The only secondary nutrient that has been deeply investigated in the recent past is sulphur, which has showed a remarkable response to fertilization in cruciferous crops such as oilseed rape (Scherer 2001; Fismes et al. 2000), potential source of several industrial products.

3.3 Fertilizers

The type of fertilizer is of minor concern for industrial crops. Both mineral and organic fertilizers are suited to the needs of this large group of crops. Generally speaking, the financial context in which they are grown does not encourage the use of expensive nutrient sources. Therefore, sophisticated fertilizers such as the "nutritional specialties" proposed by many suppliers, featuring combinations of macro- and micro-nutrients, or nutrients plus bio-stimulators, or nitrogen forms at a delayed availability (slow-release, nitrification inhibitors, etc.), seldom pay off, despite the possibility of using less fertilizer units per hectare or reducing the number of split applications. The same circumstance affects, to a lesser extent, the use of complex fertilizers vs. simple (straight) ones. This is in accordance with the world trend in fertilizer types during the past thirty years, that has been strongly influenced by economics to the detriment of complex fertilizers, the group that had, in contrast, increased faster than simple fertilizers until the mid-seventies (IFA statistics).

One last remark concerns organic fertilization that are a valuable source of nutrients as well as of organic matter for agricultural soils that are, otherwise, increasingly depleted. More to that, they supply many secondary and micro-nutrients that are virtually absent in highly-concentrated synthetic fertilizers. But the remarkable aspect is that they allow a valorisation of waste products (livestock slurries, wastes of agro-industrial, urban or other origins), both composted or not, outside the food chain. This circumstance is less worrying for the fate of these wastes than in the case of food crops, associated with the intrinsic benefit of burying organic matter into the soil, thus contributing to soil carbon sequestration.

4. Water management

Water is a crucial biological factor. Of the word's available water resource, approx. 80% is currently consumed by irrigated agriculture (Condon et al. 2004) and 40% of the world food is produced in irrigated soil (Somerville and Briscoe 2001). Projected population growth indicates 9 billion people within 2050; as such this level of consumption is not sustainable in the future as more of the water resource is expected to be used for domestic, industrial, and municipal needs. In many areas of the world, water already is a scarce resource, with respect to potential consumptions. Other regions will likely add to the list in the future, given the present trend in world population and urban growth. Crops are, likewise, constrained by moisture deficiencies; in fact, there are a few agricultural areas where potential evapo-transpiration is met by natural supplies (precipitation, water table, inflows, etc.), whereas in the rest of them, a deficit occurs. Therefore, capturing all the available water and efficiently using it for vegetable production is a moral, if not a legal, obligation. Industrial crops are even more bound to this principle: since their principal products are not for food uses, they are susceptible to be passed over, in the allocation of limited water resources.

4.1 Coping with limited water resources

Three basic mechanisms of adaptation to drought stress are present in nature (Ludlow 1989): tolerance, involving the breeding of genotypes intrinsically characterized by a better resistance, i.e., capable of photosynthesising at lower leaf-water potentials; avoidance, reflecting the plant's attitude to face the stress (osmotic adjustment, stomata closure), until the adverse condition is relieved; escape, that is, completing plant growth before the onset of severe stress, or shifting crop cycle to a season in which the stress does not occur. The three mechanisms involve different possibilities of human intervention (breeding), such as improving transpiration efficiency, i.e. the acquisition of more C in exchange for water transpired; or partitioning more of the assimilated C into the end products. Other strategies may concern a better management of water resources and of environmental potential (escape), that are not easily or timely available in practice. All these strategies should not be seen singularly; rather, they would give the best results only with complementary approaches. The ways of improving crop water use pass through the reduction of losses from soil evaporation, deep drainage and runoff, three conditions often involving a major revision of the whole cropping system (Passioura 2006).

However, the major breakthroughs in improving crop performances under dry conditions are expected from breeding. In fact, the prospects for increasing water use efficiency by improving photosynthetic efficiency at a leaf-scale are estimated greater than those for increasing the efficiency of light interception or of biomass partitioning to commercial organs, which have been widely exploited in the past. The overall breeding potential to increase photosynthetic efficiency is estimated at approx. 50%, a very high score in genetic terms (Long et al. 2006).

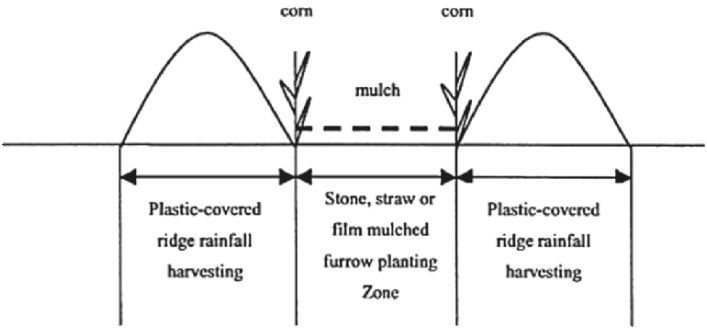


Fig. 4.5. A schematic diagram showing ridge and furrow rainfall harvesting system with mulches. Reproduced from Agricultural Water Management, 54, Li and Gong, Effects of different ridge to furrow ratios and supplemental irrigation on crop production in ridge and furrow rainfall harvesting system with mulches, 243-254, © 2002 Elsevier B.V., with permission from Elsevier.

Another possibility of increasing water availability for crops is rainfall harvesting: shaping the soil surface in ridge and furrows concentrates moisture (Fig. 4.5). In an arid region of inner China, the combination of plastic-covered ridges and mulched furrows has been capable of increasing maize yield by 60-95% in drought and average years; 70-90% in wet years and 20-30% in very wet years (Li et al. 2001), with the largest single contribution coming from the plastic cover than from the simple ridging of the soil or from the furrow mulching. Different ridge-to-furrow ratios have also been tested (Li and Gong 2002), showing an inverse relationship between average yearly precipitation and optimal value of the ratio, i.e. the driest the conditions, the largest the surface needed for water harvesting. In both experiences, water use efficiency (WUE), the parameter expressing the unit of dry biomass or commercial product per unit of water (e.g. kg m^{-3}), significantly increased with rainfall harvesting, and also supplemental irrigation, in addition to water harvesting, brought about an increase of maize yield and WUE. Therefore, the cited researches show that water harvesting not only made more water available for the crop, but enhanced its efficiency.

In contrast to this, another experience on sunflower shows that increasing soil water content at seeding through fallow management may not systematically be the best option (Aboudrare et al. 2006).

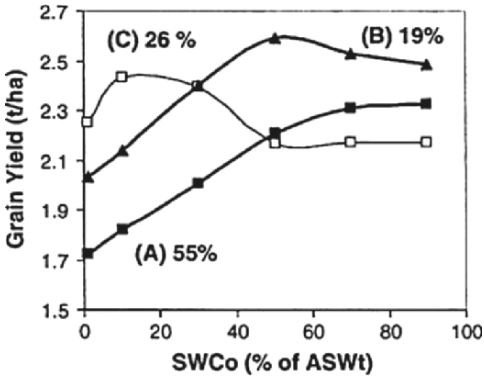


Fig. 4.6. Three types of yield response to initial soil water content (SWCo), as fraction of total available water (ASWt): simulation with EPIC-Phase (1960–1998). For each type, percent frequencies are indicated. Reproduced from Agricultural Water Management in press, Aboudrare et al. Effects of soil tillage and fallow management on soil water storage and sunflower production in a semi-arid Mediterranean climate, 1-14, © 2005 Elsevier B.V., with permission from Elsevier.

In this experience, chisel tillage proved the best compromise, within different tools and techniques, for optimising water storage in clayey soils of Morocco, among years at different amount and distribution of rains during the autumn-winter. But a high soil water reserve at seeding leads to an excessive leaf canopy at the bud stage, which in turn more rapidly depletes water and negatively affects yield and WUE, especially in a dry growing season. A partial restoration of the soil water reserve at seeding ensures, in comparison, steadier yields, as shown by a simulation run over the years, where a cumulated frequency of 45% of maximum yields is achieved with an initial moisture not exceeding 50% of total available water (Fig. 4.6).

So, the direct harvesting of water and the indirect techniques to store it may have contrasting effects, which need to be carefully evaluated before setting out for any programme. In the case of water harvesting, a considerable drawback is represented by the large request of labour for carrying it out, although in developing countries the system is worth consideration, if it is deemed capable of supporting yields.

4.2 Irrigation

The supply of water is basically conditioned by its availability at the needed time, and by the economic convenience in using it. The latter, in turn, depends on the cost of water and of its application, on crop physical responsiveness to irrigation and on crop selling price. It is easily understood that any failure in the chain of supply/valorisation of water undermines the financial return of irrigation.

Water use efficiencies of either the total amount consumed by the crop (WUE) or that supplied through irrigation (IWUE), are good estimates of crop overall efficiency and responsiveness to irrigation, respectively. Irrigation is susceptible of enhancing growth, raising yields, often at the expenses of WUE. Therefore, the conditions must be sought, in which this circumstance takes place to the least extent. Deficit irrigation, a supply of water that is only a fraction of the evapo-transpiration deficit, may improve yield and also WUE, especially if applied in critical phases of crops growing in severe water stress (Xue et al. 2006; Tavakkoli and Oweis 2004). Since in dry areas the rotation is often reduced to the fittest crops, which in many cases means a monoculture of winter wheat, widening the rotation may improve WUE, along with the duration of crop coverage during the rainy season and the amount of rain intercepted by crops (Huang et al. 2003).

In grain sorghum, IWUE has been shown to decline at increasing irrigation volumes in lysimeter studies (Tolk and Howell 2003), whereas trials in the open field have showed an opposite trend (Farré and Faci 2006). Grain sorghum has often proved a good alternative to maize in water-limiting environments, thanks to higher WUE and IWUE, harvest index, soil-moisture depleting potential (Farré and Faci 2006) (Fig. 4.7), and water-table exploitation (Sepaskhah et al. 2003). Grain sorghum is also a potential energy crop, but more-dedicated ones are sweet and fibre sorghum, of the same species as the grain type (*Sorghum bicolor* (L.) Moench), but at high vegetative growth and comparably-lower grain yield. In Southern Italy (Mastrorilli et al. 1999), sweet sorghum has proven more sensitive to early water stress, during the early ("leafy") stages of plant growth, than in the later ("stem") stages: over three years, the early, temporary stress significantly affected growth, final yield (-34% dry biomass than the well-watered control) and WUE (-17%), whereas the late stress, at a comparable water consumption, brought about lower, insignificant variations: -11% in yield and +6% in WUE. In the wetter environment of Northern Italy (Amaducci et al. 2000), fibre sorghum did not take advantage of irrigation, although it was by far the highest-yielding crop among three other fibre crops (hemp, kenaf, fibre maize), whose two (kenaf and fibre maize) were significantly enhanced by irrigation. The insignificant and positive effects

of irrigation on the dry matter yield of, respectively, fibre sorghum and kenaf were also confirmed by other experiences in sandy soils under similar wet conditions (Monti et al. 2002).

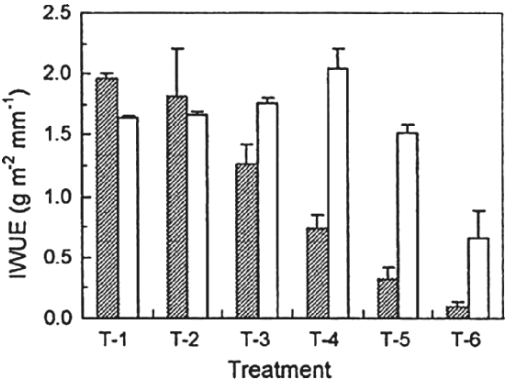


Fig. 4.7. Irrigation water use efficiency (IWUE), expressed as the ratio of grain yield to seasonal irrigation applied, for the different irrigation treatments in maize (full bars) and sorghum (empty bars). Bars represent standard errors. T-1 to T-6 represent decreasing irrigation treatments. Reproduced from Agricultural Water Management in press, Farré and Faci, Comparative response of maize (*Zea mays* L.) and sorghum (*Sorghum bicolor* L. Moench) to deficit irrigation in a Mediterranean environment, 1-9, © 2005 Elsevier B.V., with permission from Elsevier.

Both sweet sorghum with late water deficit in the former research (Mastrorilli et al. 1999) and the rainfed fibre type in the latter ones (Monti et al. 2002; Amaducci et al. 2000) attained average dry-matter yields of 26-28 Mg ha^{-1} in trail plots, associated, in the previous research, with a WUE of about 6 g kg^{-1}. The combination of the two figures well describes the species' remarkable potential.

Miscanthus (*Miscanthus x giganteus* Gref et Deu) is another biomass crop investigated under irrigation. Miscanthus is a C_4 grass like sorghum, but is a rhizomatous perennial. In a Mediterranean environment of Central Italy, irrigation showed a positive interaction with N fertilization (Ercoli et al. 1999): in fact, at no nitrogen supply, irrigation did not raise biomass yield, which was, conversely, enhanced at full N rate (200 kg ha^{-1}). The positive interaction between nitrogen and irrigation almost doubled net energy yield (output – input), given that the calorific value was uninfluenced by the two factors. In contrast, energy efficiency (output/input) was more than halved in the rainfed crop by the input of nitrogen, and significantly fell also in the irrigated crop. The positive effect of irrigation on dry matter yield of miscanthus was also ascertained in North Italy under a wetter conditions, yet no significant interaction with nitrogen dose was observed in this case (Monti et al. 2002). In a pot experiment, three Miscanthus species

were compared under water stress, in order to investigate WUE and biomass partitioning (Clifton-Brown and Lewandowski 2000). A *M. sinensis* hybrid offered a better resistance to leaf senescence than *M.* x *giganteus* and *M. sacchariflorus*, thanks to a reduced leaf conductance. Because of that, WUE on the total biomass did not significantly vary among the three genotypes, whereas WUE on the stem component showed differences in dry matter partitioning, to the advantage of *M. sacchariflorus* over the other two genotypes.

A non-gramineous industrial crop widely investigated as it concerns water relations is sunflower. The crop is a profligate water user (Connor and Sadras 1992), although it has a deep rooting and a good ability to tap on ground water. It is most drought-sensitive at the beginning of the reproductive stages, which is confirmed by a recent research in a Mediterranean environment (Göksoy et al. 2004): irrigating only at flowering showed by far the highest IWUE, compared to irrigating at heading, milking and at various combinations of the three stages. A more-intensive irrigation programme further enhanced seed yield, at the expenses of high amounts of water. In another experience in a semi-arid environment (Gajri et al. 1997), deep tillage (0.4 m) and/or mulching helped the crop in efficiently using soil water, which pressed irrigation to show a stronger response in conventional tillage (0.1 m depth). The crop appears, anyway, not suited for very dry soils and climate, since the volumes needed to optimise yield (350-800 mm) seem too high for an economic use of water.

The application of crop simulation models to sunflower (Rinaldi 2001; Rinaldi et al. 2003) once more showed that the highest economic return is attained by deficit irrigation, involving either irrigating (200 L m^{-2}) only at bud flower stage (EPIC model), or irrigating whenever soil water content passed below 40% of total soil water capacity (OLICROP-SUN model).

Irrigation and advanced seeding influenced also fatty acid composition of high-oleic sunflower, with a decrease in oleic and an increase in linoleic acid, an unfavourable combination likely due to the effect of lower temperatures in the early phases of seed development, enhancing the enzymatic transformation of the former into the latter (Flagella et al. 2002).

Irrigation was also found to consistently increase the above-ground structural parts in the two inulin crops chicory and Jerusalem artichoke (Monti et al. 2005 a, b; Schittenhelm 1999). However, dry biomass accumulation did not run in parallel to the fructan storage. The only significant effect of water regime was to speed up the accumulation of fructan in chicory, and to delay the tuber formation and degree of polymerisation in

Jerusalem artichoke, probably related to the sink-to-source ratio (Monti et al. 2005a; b).

4.3 Irrigation management on a large scale

A few words need to be spent on the management of irrigation at the district/basin level, implying an articulate reasoning on crop needs, water availability, and other related issues. Simulation models and remote sensing play an important role in the subject.

The first step is the assessment of water needs, i.e., the imbalance between precipitation and crop evapo-transpiration (ET). The possibility of determining ET fluxes through satellite sensing of canopy biophysical properties, integrated by agro-meteorological information, has been successfully proved in a very homogeneous district (Consoli et al. 2006), but the same result would be less-easily achieved in areas characterized by a mixed agricultural landscape. In such areas, reference ET, simulated by several equations or methods, should be the base for assumptions and adaptations in order to better fit the data to the territory.

The second, capital step is the management of water resources. To this aim, geographical information systems (GIS) are widely employed, in association with crop modelling. An example of a good integration of the two tools in the frame of best management practices for irrigation and nitrogen fertilization, applying multi-criteria analysis to a set of agro-environmental indicators, showed that water-flow control was the critical point for reducing pollution in a drainage basin of Northern Italy. Controlling it through a better irrigation tuning enabled significant reductions in nitrogen leaching, while improving crop yields (Morari et al. 2004). Other GIS applications are aimed at supporting the implementation of improved farm irrigation management, as related to water saving and salinity control (Fortes et al. 2005), and for the development of irrigation scenarios at different scales, according to spatial variation, climatic and management conditions (Todorovic and Steduto 2003).

The remote control of large irrigation networks is a rising possibility with WLAN (wireless local area network) technology, associated to solar panels to power the system. In one such example (Damas et al. 2001), the division of a 1500-ha area into seven sub-regions, monitored and controlled by inter-communicating sectors subjected to a central unit, enabled programming/carrying out of the irrigation shifts, controlling pumps/valves works and reservoir levels, while claiming a significant (30-60%) possibility of water saving.

The last capital issue at basin level is the allocation of water for irrigation vs. other uses. The problem, as Lankford (2004) observed, is that the irrigation designs laid out without sufficiently accounting for alternative uses of water in semi-arid environments, tend to over-prioritise water for irrigation systems ("irrigation centred"), at the expenses of re-allocation to other uses. Focusing, instead, on the river basin in a "water-resource-centred" approach, is argued to better comply with the multiple interests involved in the use of water.

A similar idea is echoed by Rosenzweig et al. (2004), who analysed the implications of changes in crop water demand and water availability for the reliability of irrigation systems, by linking climate change scenarios with hydrologic, agricultural and planning models, in five major agricultural regions around the world. The simulation showed that only one study area (Brazil) can readily accommodate an expansion of irrigated land under climate change; three others (Northern Argentina, the Danube Basin and the US) would suffer decreases in the reliability of irrigation; the last case, Northern China, already experiences a serious lack of water at present. It is, therefore, concluded that even in the relatively water-rich areas, changes in water demand due to the climate and to increased demand from urban growth will require timely improvements in crop cultivars and water management, in order to comply with the projected scenarios.

5. Crop protection

Crop protection from the competition of weeds and from the biotic stresses caused by pests and diseases is too vast a subject to be acceptably discussed in a limited space, since a few guidelines may be found in the huge combination of crops per weeds, pests and diseases. Nevertheless, some trends and common behaviours may be outlined, in tackling these problems in a modern crop management.

It is almost needless to repeat that industrial crops have to be cheap in cropping and efficient in the use of inputs, above all in the conversion of energy. More to that, the sensorial quality of their products, meant as agricultural raw materials, is of the least importance. It follows that only the biotic stresses/competitions that are seriously threatening yield or industrial quality should be taken into consideration within a programme of crop protection.

5.1 Assessing the damage

A brief outlook on the subject may begin with the assessment of the yield-loss potential (no-control scenario) compared to the actual loss, in some prominent arable crops (four cereals, soybean, sugar beet, potato and cotton) in seventeen agricultural regions around the world (Oerke and Dehne 2004). The loss potential varies from less than 50% (barley) to more than 80% (sugar beet, cotton). The actual loss, given the control measures normally deployed, is consistently lower and unrelated to potential loss; it varies from 25-30% of sugar beet, barley, soybean, wheat and cotton, to 35-40% of maize, potato and rice. The efficacy of crop protection (mechanical, physical, chemical means) is, therefore, higher and more consistent in cash crops (sugar beet, cotton), whose relevant loss potential is quite well controlled (60%) by a more intensive protection programme; conversely, the efficacy is modest and more fluctuating in the rest of the crops (average 46%), where a lower loss potential is associated with tighter profits, hampering the implementation of a better strategy. Among the sources of yield loss, weeds have the highest potential (32%), followed by animal pests and pathogens (18% and 15%, respectively) and by viruses (1-3%); but since weeds can be controlled through mechanical or chemical means, average efficacy in weed control (68%) is considerably higher than that in pest and disease controls (39% and 32%, respectively), which rely more heavily on pesticides.

In this picture, industrial crops are not included, apart from cotton. It may be sensed, anyway, that their global behaviour should resemble that of cereals, themselves being grown for industrial end uses, sometimes. In other words, the group is globally expected to behave like crops less-severely impaired by the complex of pests, diseases and weeds, but at the same time less prone to efficient control measures. The reasons are the intrinsic financial constraints, but also a lower number of registered active ingredients, and a frequent lack of information on pest-and-disease epidemics in these specific crops.

5.2 Integrated crop protection

Given these limitations, an integrated approach to crop protection, combining chemical active ingredients with agronomic and genetic tools, is the one that best fits the majority of industrial crops. This same approach is widely followed in food crops, too, where a reduction in the use of pesticides is equally sought. The critical point of its implementation is acknowledging the role of the agronomic factors, in order to harmonize them towards the desired aim. Therefore, the recognition of the effects carried

out by agronomic practices, especially in the area of crop rotation and soil tillage, is the key to a successful exploitation.

Examples of such effects and of the subtle mechanisms involved are offered by Meynard et al. (2003), who showed how cropping systems in the case of winter wheat have a large effect on the size of the primary inoculum of eyespot (*Pseudocercosporella herpothricoides*) and on its localisation at the soil surface, directly related to the extent of disease symptoms: keeping the residues of a previous wheat crop as far from the surface as allowed by crop sequence and soil tillage, helps to significantly reduce the attack, whereas a careless management, e.g. ploughing twice since the previous wheat crops, brings more inoculum to the surface and enhances disease severity. Other effects, still reported by Meynard et al. (2003), concern: the development and spread of epidemics, with an example of the slowing of grey mould of vines (*Botrytis cinerea*) thanks to several indirect practices in vineyard management; the coordination of the life cycle of cultivated plants and related parasites, showing both positive (oilseed rape) and negative (wheat) effects resulting from advanced seeding, depending on the specific, respective parasite (black leg and eyespot) and on the associated time of contamination (spring and autumn, respectively); at last, the disrupted ecological equilibrium within soil microflora, either favouring or disfavouring the pathogens, in a complex study case on take-all of wheat (*Gaeumannomyces graminis*) in various patterns of set-aside management.

Other examples may be found in literature, but the point is that the cumulated experiences on the combinations of single pests (diseases, weeds) and crops should be sufficiently advanced for the build-up of decision support tools for a better design of cropping systems, a circumstance that is now achieved only for a few adversities in a limited number of crops; in fact, the crop-protection support systems currently in use are more focused on the use of existing pesticides, than on integrated protection management (Murali et al. 1999). In the rest of cases, the maturated experiences on synergies/antagonisms and the general knowledge of basic agronomic effects may be played within the farmer's sensitivity and habits. The fact enhances personal abilities but undermines the possibility of a consistent support to cropping choices, which reflects on the quality of cropping and, eventually, on the consistency of yields.

5.3 The contribution from breeding

The contribution of breeding to weed control and crop protection, including GM crops, is not negligible, although it may only be hinted at, in the present outlook. The resistance to pathogens is a constant effort in many breeding programmes: improved varieties are continuously being released

with strengthened characters of resistance to specific parasites. Examples are present also in industrial crops, such as in sunflower, with the added resistance to powdery mildew in existing hybrids, significantly reducing disease symptoms (Laureti et al. 2006).

Genetic modifications are a powerful tool in the farmer's hands to cope with biotic adversities. Some industrial crops already display genetically-modified varieties, in the frame of resistance to non-selective herbicides (e.g. oilseed rape) and to insect pests (e.g. cotton). The much-debated worldwide acceptance of GM crops is somehow slowing their progress, but other constraints need not be forgotten, such as the fact that crops of limited worldwide diffusion are not likely to be ever subjected to these transformations, and that the use of GM crops, such as the herbicide-resistant ones, raises concern not only among environmentalists, but also for some agronomic drawbacks, such as the strong selective pressure imposed on weed flora, possibly enabling it to overcome herbicide control.

With this remark, GM crops as well as traditional genetic resistance are to be viewed as complementary tools in crop protection, not as the ultimate remedy to plant biotic adversities.

6. Precision agriculture

Precision agriculture is a subject of increasing interest, encompassing all the previously-discussed categories of crop management. It is seen as the new frontier of agronomy by some scientists; others more realistically view it as a tool susceptible of improving the ways of tackling existing problems.

The concept of precision agriculture lies on the technology of the global positioning system (GPS), a method for soil-surface locating through satellite sensing, with a remarkable accuracy (0.05-0.1 m). GPS-provided harvesters equipped with on-the-go yield monitoring produce yield maps, showing the extent and the pattern of yield variation within the field. Once reasons have been identified for the differences in yield, site-specific application of nutrients, active ingredients, seeding, water and tillage (Fig. 4.8) can help to overcome the problems or to cope with them, while at the same time avoiding the contradiction of uniform treatments applied to variable soil conditions.

This is in principle; in practice the problem is that, since the act of yield mapping, it is quite easy to incur in errors, miscalculations, etc., leading to incorrect assumptions for the decision to be taken. More to that, a whole generation of sensors is needed for both yield-monitoring at harvest, and continuous sensing of soil/crop properties during crop cycle, that are still

being evaluated and improved, according to the evolution of technology and to the results in field tests.

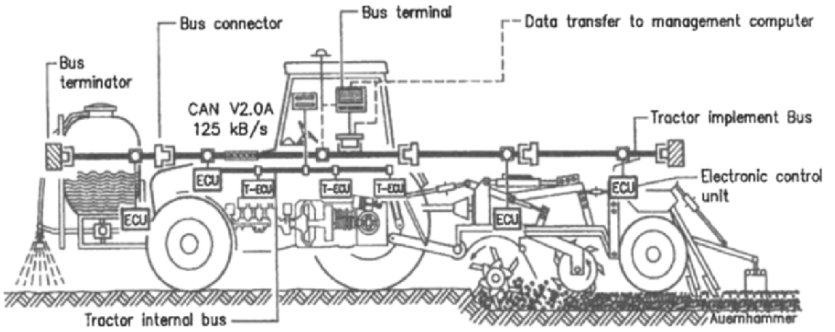


Fig. 4.8. The 'Landwirtschaftliches BUS-System (LBS)', an example of communicating outfit between tractor, equipment and farm-management system, catering to the needs of small-scale European farmers. Reproduced from Computers and Electronics in Agriculture, 30, Auernhammer, Precision farming - the environmental challenge, 31-43, © 2001 Elsevier B.V., with permission from Elsevier.

The combined complexity on these two sides of monitoring, associated to the field size, to the cost of instruments, and to the overlapping and sometimes prevailing effects of unpredictable climatic conditions on yield, considerably slows the progress of precision agriculture. A short review of the recent literature may, as in other cases, reveal pending problems and highlight current trends, seen in the perspective of industrial crops.

6.1 Yield mapping

The problem of dealing with the errors in yield maps has been recently investigated by Robinson and Metternicht (2005), who managed to rectify them. In their research on winter wheat in Australia, all the sources of error (unknown harvest width, time-lag in grain-yield sensing, inappropriate GPS recording, yield surges, and other outlying values) made up 17% of the total data-set acquired on a 96-hectare surface; its elimination resulted in a substantial reduction in the amount of uncertainty. The accuracy of spatial-interpolation techniques was assessed over the whole surface by minimizing the differences between true and interpolated values (Root Mean Square Error parameter), although it was argued that the resulting map of the area, used to assist in future crop management, should not be based only on RMSE values, but also on the degree of smoothing and data aggregation that is desirable and allowable by the filtered data-set.

Yield maps are also the subject of hot debate and criticism. Assumptions based on winter-cereal yields, for instance, do not easily apply to crops of different family, cycle, habit, like sugar beet (Jaggard et al. 2000). In quite-uniform cropping systems, such as in cereal-dominated rotations, there is compensation in the spatial distribution of crop yields over the years, so that the spatial variation reduces with time (Godwin et al. 2003). Even so, anyway, maps of previous crops' yields did not prove a useful basis for assessing a strategy of variable nitrogen rates in the cited experience, compared to aerial photography of the crop in the actual growth conditions.

The problem of the yields recorded on the same field from different crops succeeding in time does not apply to perennial crops. In such case, maps may highlight soil physical and chemical characteristics associated with yield (Di Virgilio et al. 2006), whose some are susceptible of being improved during the years, to the benefit of cumulated yield.

This case, anyway, confirms the role of yield maps as more suited for directing soil sampling to identify potential constraints, than for a direct strategy of input-adjustment in the following crop.

6.2 Real-time monitoring

The second category of monitoring, devoted to "real-time" sensing and decision-making, is a more promising field of interest, as a larger number of scientific references testifies. The tools and the technologies employed in this direct probing of soil or crop properties are the core of the problem.

6.2.1 Soil sensing

Adamchuk et al. (2004) recently reviewed on-the-go soil sensors, grouping them in six categories: i) electrical and electromagnetic sensors, measuring electrical resistivity/conductivity, capacitance or inductance as affected by soil composition; ii) optical and radiometric sensors, using electromagnetic waves to detect the level of energy absorbed/reflected by soil particles; iii) mechanical sensors, measuring forces resulting from a tool engaged with the soil; iv) acoustic sensors, quantifying the sound produced by a tool interacting with the soil; v) pneumatic sensors, assessing the ability to inject air into the soil; vi) electrochemical sensors, using ion-selective electrodes and transistors that produce a voltage output according to selected ions (H^+, K^+, NO_3^-, Na^+, etc.). Different soil properties are targeted by the various methods: primarily texture, organic matter, compaction/bulk density, pH and nitrate (Table 4.4).

The only sensors widely used now are the electrical and electromagnetic ones, that also have the wider spectrum of activity over soil properties. At the same time, electrochemical sensors are a promising tool to directly evaluate soil chemical fertility (pH, nutrients).

Table 4.4. Soil properties targeted with various on-the-go soil sensing methods. Adapted from Computers and Electronics in Agriculture, 44, Adamchuk et al. On-the-go soil sensors for precision agriculture, 71-91, © 2004 Elsevier B.V., with permission from Elsevier.

Soil Properties	Electrical, electromagnetic	Optical, radiometric	Mechanical	Acoustic, pneumatic	Electrochemical
Soil texture	X	X		X	
SOM[a]	X	X			
Soil moisture	X	X			
Soil salinity[b]	X				X
Soil compaction[c]			X	X	
Depth variability[d]	X		X	X	
Soil pH		X			X
Residual NO_3, TKN[e]	X	X			X
Other macro-nutrients[f]					X
CEC[g]	X	X			

[a]Soil Organic Matter (or soil organic carbon).
[b]Or sodium content.
[c]Or bulk density.
[d]Depth of topsoil or hard pan detection.
[e]Total Kjeldahl Nitrogen.
[f]Potassium content.
[g]Cation Exchange Capacity (and other buffer indicators).

The constraints that remain to be overcome are the response lag, still inhibiting a direct on-the-go adjustment of fertilizer or lime application, and the need to calibrate the analytical system like in a conventional laboratory, prior to operating. More to that, in a subsequent study (Adamchuk et al. 2005), the precision of eight electrodes, assessed in a comparative trial, remarkably decreased for the tested parameters in the order: pH, K, nitrate-N, Na, showing the need for additional work, in order to ensure a good reliability.

Among electrical and electromagnetic sensing, the measurement of apparent soil electrical conductivity is mentioned as the most reliable and frequently used to characterize the spatial variability in soil edaphic properties (Corwin and Plant 2005). Its greatest potential is deemed by the

authors to provide reliable information for directing soil sampling to better identify and characterize spatial variability of parameters influencing crop yield.

6.2.2 Crop sensing

Hints of the direct measurements made on growing crops, in order to spatially-modulate inputs, have already been given in the nutrients and water sections, where ground and remote optical sensing of crop nutritional status and satellite sensing of ET fluxes were discussed. More to that, it may be said that, according to Scotford and Miller (2005), the optical techniques of spectral reflectance seem best suited for aiding decisions in crop nutrition until the crop attains full soil cover, yet reveal limitations as to weed and disease assessment, as well as to seed-rate adjustment on the base of seedbed quality. Likewise, spectral reflectance is unlikely to be used for measuring soil properties that are better evaluated by other means.

Crop sensing may be viewed as the most advanced sector within precision agriculture; at the same time, it seems more promising in combination with other tools, than alone. In fact, the association of a simulation model (CROPGRO) and aerial sensing (analysis of the Normalized Difference Vegetative Index) was successfully tried (Basso et al. 2001), enabling the identification of spatial patterns of soybean growth and the prediction of yield variability, thus fostering the idea that a zone-specific management can be developed, based on the association of the two techniques.

Auernhammer (2001), too, pointed out as the two strategies for implementing both fertilisation and protection, yield mapping integrated by soil sampling and crop sensing, may in fact converge into a systematic approach, where the potentials of the two means are enhanced, while the weak points are reciprocally compensated. In concept, this corresponds to passing from farming by balance (mapping approach) or by growth (real-time approach) to farming by sustainability (Fig. 4.9).

6.3 Other issues in precision agriculture

The site-specific management of crop nutrition remains the issue of major weight in the move towards precision agriculture, according to Auernhammer (2001). The resulting advantage is, therefore, quite small, and it is perceived that the technique will gain importance only when collateral benefits, such as reduced environmental burden and increased flow of information, are acknowledged as an added value. As to this, an environmental benefit of primary importance has been shown by Sehy et al. (2003), who observed how site-specific fertilizer treatments (method of

previous-yield maps), supplying less N fertilizer to low-yielding areas, resulted in 34% less releases of the highly-noxious nitrous oxide from the soil, while not depressing maize yield.

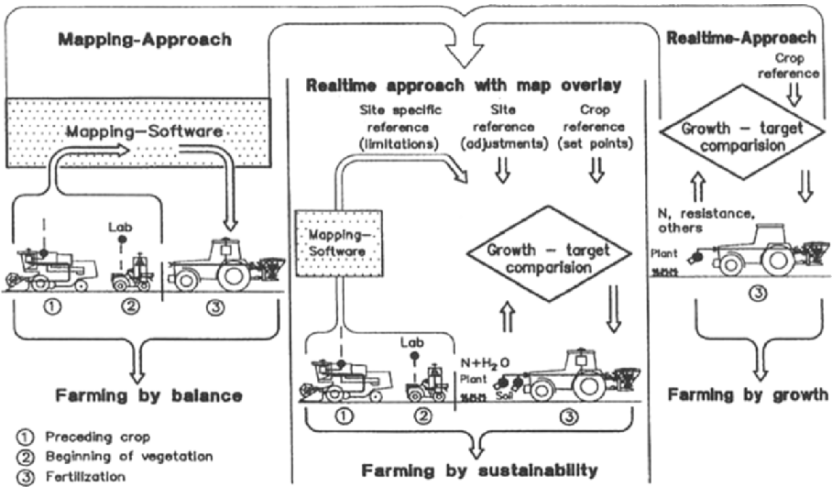


Fig. 4.9. Systematic approaches for the implementation of site-specific fertilisation. Reproduced from Computers and Electronics in Agriculture, 30, Auernhammer, Precision farming - the environmental challenge, 31-43, © 2001 Elsevier B.V., with permission from Elsevier.

The issue of an adequate return from the adoption of precision-agriculture techniques is only second in importance to the problem of which system to choose. To this aim, Godwin et al. (2003) have laid out a practical guideline in the shape of a flow chart, based on their experience on nitrogen fertilization of winter cereals in the UK. Five stages are identified: i) an appraisal of within-field variation; ii) the quantification of the threshold yield increases needed to justify the investment; iii) understanding the causes of variability and identifying management zones; iv) addressing fundamental management practices (e.g. other nutrients; non-nutrient limiting factors) prior to N variable application; v) the real-time management of variable nitrogen fertilizer for optimising economic yield. The five-step process allows to correctly evaluate all the factors implied in precision cropping, in order to implement it on a sound basis.

On conclusion, precision agriculture is a domain of rapid scientific-technical development, where today's knowledge, assumptions and results may soon be obsolete. Basically, it does not contribute new concepts to agronomy, whereas it promotes a re-organisation of agricultural practices, on the grounds of a better compliance with the characteristics and the potential of the cropping site.

7. Harvest, conditioning and storage

Harvest and subsequent conditioning/storage of crops for industrial end uses is another category that cannot fit simplification. The plant organs of industrial interest are the most varied: seeds, possibly with the associated fruits; stems; the whole above-ground biomass; or even roots or underground storage organs. According to this, a first subdivision may be traced between grain crops for industrial end uses and "biomass" ones, meaning for the latter those crops where the portion of commercial interest is represented by vegetative organs.

7.1 Grain crops

Harvest, conditioning and storage of common grain crops for industrial end-uses, such as many cereals and oilseeds, are widely known and represent a state-of-the-art that in the present chapter seems needless to discuss.

Maybe the only exception is represented by oilseed rape (*Brassica napus* L.), a cruciferous in which harvest time and method are basic determinants of crop yield. In fact, Weiss (1983) reported that yield losses may be up to 30% due to an incorrect use of harvest machinery. Oilseed rape is considered mature when pods become yellow and seeds very dark with a moisture of about 15%. This usually occurs about 200-230 days after sowing autumn crops and 100-130 days after spring ones, but the species is also characterized by a progressive bottom-to-top ripening, associated with the risk of pod-shattering. Given this constraint, the optimum harvest window may reduce to only one week (Weiss, 1983). The best technique, according to the species' biology, consists in cutting and windrowing the stems when pods at mid-height become yellow, then threshing with a pick-up combine when full maturity is achieved. It is essential that cutting occurs at a seed moisture not exceeding 35%; operating below 20% and over 45% will cause significant losses, respectively on yield (pod shattering) and quality (oil and protein content). The alternative is to combine the standing crop, at a more-advanced maturity. Combining either directly or from windrows is the single operation that causes the largest losses: the small size makes the rapeseeds easy to disappear at the cutting edge or through the smallest cracks and holes in the machines.

After combining, rapeseeds have a moderate-to-high moisture (approx. 15-25%), requiring a rapid air-drying (to 7-9%) to be carried out, in order to avoid seed deterioration: in fact, only 24 hours are needed to start seeds deterioration at 20°C and 18% moisture. Besides, a number of impurities are normally present with the seeds, therefore re-cleaning is often necessary to prevent contamination and heating in bulk.

7.2 Biomass crops

7.2.1. Herbaceous biomasses

In general, proper biomass crops are tall and stiff (e.g. giant reed, Miscanthus). As such, standard mowing machines for grass and forage crops do not generally work well with them and need to be adapted in order to successfully perform, though in some cases this may be untrue, as some biomass crops were found to be very suited to common forage machines without adaptations (Venturi et al. 2004; Hadders and Olsson 1997). For example, in Miscanthus the 'Kemper' mowing attachment used for silage maize should be row-independent and work rather high to prevent jamming. In this case, most biomass could be lost unless a special mowing device for low-cutting is added (Lewandowski et al. 2000).

Various harvesting chains have been tested so far: mowing and chopping, mowing and baling; mowing and bundling, etc.. If feasible, baling should be usually favoured, as it leads to a more compacted material at higher energy density. For example, the bulk density of round and square bales of switchgrass were from 112 to 141 kg m^{-3} of dry matter, while chopped material did not reach 70 kg m^{-3} (Venturi et al. 2004). Moreover, the choice of the harvest method also influences the costs per energy unit for the subsequent storage and transport. As for this, Venturi et al. (2004) calculated that 1904 and 2131 MJ m^{-3} are transported, using round or rectangular bales, respectively. Hence, with an available volume of approx. 30 m^3 (truck plus trailer), 57.1 and 63.9 GJ can be delivered each time. As for biomass sorghum, harvest is further complicated by the fact that problems associated with sweet sorghum are different from those of fibre sorghum, the first having much more soluble sugars in the stem.

In the Mediterranean area, the best available technique may be cutting and conditioning in one pass (counter-rotating drums), then windrowing to air-dry the material and finally baling it up by means of a high-pressure-chamber baler. However, meteorological constraints frequently delay the windrow drying, thus favouring fermentation processes while increasing biomass losses, especially in sweet sorghum. Sugar cane harvest machines can be very suited for sweet sorghum as well, although they generate cut stems of about 0.3 m length which can be stored no longer than 3 days prior to processing, making them of limited practical use.

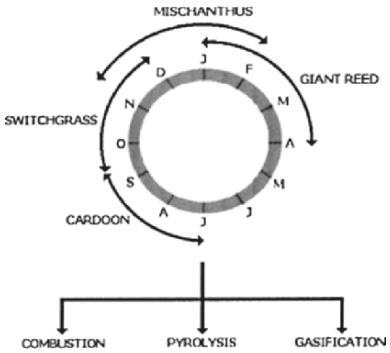


Fig. 4.10. The organisation of the supply to power plants, by means of biomass crops harvested around the year. M. Christou, internal meetings, EU Project Bio-energy chains from perennial crops in South Europe (2001-2005).

As for the relationship between harvest time and biomass quality, generally speaking, the later is the harvest, the lower are moisture and mineral content, thus enhancing the quality of the harvested biomass. However, there is a trade-off, since postponing the harvest to the springtime has a cost in terms of yield, mostly explained by leaf losses (Huisman and Kortleve 1994). For many biomass crops, the harvest window may extend from early autumn to the following spring, looking for the best compromise between full exploitation of the growing season's potential and acceptable operativeness at harvest. There are also exceptions of crops performing a rapid growth before the onset of drought, like cardoon (*Cynara cardunculus* L.), which in turn enable a better round-the-year supply of "fresh" biomass to power plants (Fig. 4.10).

For the rest of biomass crops, whenever the steadiness of supply can be achieved through storage, harvest is more often performed in spring, in order to collect a dry standing biomass, i.e. with the maximum uncut dry matter. Nonetheless, in spring the optimal time for harvesting can be very short because of the risk of heavy rainfall, associated to the need of sowing the following crop (annual biomass crops), or to the beginning of plant regrowth (perennial crops), that in temperate regions usually occurs in early April. Alternatively, many biomass crops may be harvested twice a year: early in the season (during summertime) at top moisture, followed by field-drying; then in winter, exploiting natural freeze-drying from the season's frosts. The outcome in terms of yield, quality and economic profitability is still under evaluation. Artificial drying or ensiling does not seem worthwhile for biomass crops at energy uses.

There are unavoidable losses of dry matter during harvest and storage of plant material (Moser 1980), either one or the other being prevalent depending on climate conditions, harvesting method, biomass composition, the last term being especially related to moisture content. For example, Rees (1982) estimated the total dry matter losses up to 30% with most of it resulting from plant respiration during drying; Coble and Egg (1987) showed total harvesting losses up to 40% for sweet sorghum, due to unrecoverable biomass.

Biomass losses may be also affected by the shape of the harvested biomass: it was shown that an almost two-fold deterioration of sweet sorghum occurred in the surface layer for outdoor storage over six months (Coble and Egg, 1987). These results were corroborated by Bledsoe and Bales (1992): the authors concluded that a wetter biomass was the main cause for the losses, the upper 0.15 m being more weathered than inner parts. Therefore, all the techniques which decrease rain seeping or allow a better drainage may strongly reduce storage losses (Russell and Buxton 1985).

At last, some crops such as switchgrass have been found to be less susceptible to biomass losses both during harvesting and storing. For example, Sanderson et al. (1997b) estimated biomass losses during baling of switchgrass at 1 to 5%, while dry matter losses after 12 months of storage were only 5 to 13%.

7.2.2. Short rotation coppice

An even more substantial research and development effort has been carried out in the mechanisation of the short rotation coppice (SRC) during the last decades. However, some issues still persist, mainly concerning harvest and the associated downstream processes that are clearly the most significant points in terms of overall effectiveness of the enterprise. Typically, the most suitable SRC within Europe are types of poplar (*Populus* spp.), willow (*Salix* spp.) and Eucalyptus, the last being especially adapted to Southern Europe. Harvesters can synthetically split into two categories: i) stick harvesters, i.e. machines collecting long sticks or shoots; ii) cut and chip harvesters, i.e. those cutting and chipping the crop in a single pass. Both may have advantages and disadvantages. For example, stick harvesting is generally cheaper and has lower biomass losses; in contrast, it needs large storage space: indeed the low bulk density of sticks make the transport expensive in conventional systems. At present, cut and chip harvesting seems favoured by the larger operations feeding chips to power-generation plants (Culshaw and Stokes 1995). This could be mainly attractive where the chips go straight from the field to a combined heat-and-power plant which can burn them within a few days from harvest.

Another problem associated with perennial biomass crops, both grass and tree ones, is that, since they are usually harvested in the winter months when soils are often wet, soil compaction becomes an important issue with respect to harvesting technique; in fact, since there is no opportunity to carry out cultivation operations to repair any soil-structure damage over the plantation's life. Soil compaction can be minimized by using large tyres, keeping machines weight as low as possible and by reducing unnecessary traffic across the field.

7.2.3. Fibre crops

Prominent fibre crops (cotton, flax) already own well-developed harvest-and-conditioning chains with dedicated machinery, fine-tuned logistics etc., the occasional difficulties arising from unfavourable weather, crop faults (e.g. biotic stresses, non-uniform maturity) or other causes.

In contrast to this, hemp was a traditional fibre until it almost disappeared at the advent of artificial fibres, in the second half of the twentieth century. The crop is now undergoing a process of re-introduction through a better valorisation of its fibres. In this frame, the development of a suited harvest-and-conditioning chain is to be thoroughly re-designed, according to the end use of hemp fibre: for example, it must be taken into account whether or not to use core and bark separately, whether or not to maintain fibre parallelism, the fibre length demanded by the end-user, etc.. However, not only the end use but also the processes following harvest are crucial for the choice of the machinery; for example, if scutching is done before or after retting, fresh or dry stems will be used.

For textile destinations, machines for flax could be used, apart from the fact that their hackling systems are dimensioned for fibre ribbons varying from 0.9 to 1.1 m, which are substantially smaller than that of hemp. However, the construction of hackling systems for the processing of longer stems, though already technically feasible, is likely too expensive because of the currently-limited hemp market. It is therefore necessary: i) to keep the hemp plants short ("baby-hemp"), or ii) to cut the stems into two or more pieces, no longer than 1.2 m. The first option can be achieved by following appropriate crop techniques such as using early varieties, with high plant density (Amaducci et al. 2002), or curbing the growth by means of chemical treatments (i.e. the "standing dead"). However, because of the chemicals used, the standing dead is in contrast with ecological trends; moreover, it has received a poor rating due to low fibre quality. Alternatively, scutching and hackling could be achieved by cutting hemp stems into smaller sections. For that purpose, the crop is grown in the traditional way, whereas major changes must be applied to the harvester (Amaducci 2005), according to prototypes developed in a recent EU Project (HempSys).

Once harvested, successive phases (swaths turning, baling, fibre separation, etc.) can be performed by flax-dedicated machines without substantial modifications.

To cut the plant, different mowers can be used (see Venturi 2004 for review). For sparse crops with thick stems, rotary drums mowers with conveyor belts tend to work best, bashing the stems before cutting them, and with lateral separators to avoid wrapping of the fibre around the cutting organs. An interesting variant could be the use of a mower-conditioner that crushes the stems, removing part of the core, thus facilitating field-drying. Interesting outcomes were also found by combining mower and on-field decorticator: the mower leaves stems in swaths, followed by a decorticator with a pick-up that collects the cut stems for immediate decortications (Venturi 1970).

8. Conclusions

Agronomy and management of industrial crops are an area of some technical/scientific delay with respect to food, cash and forage crops. The reasons are the novelty of many such crops, and the research focus that has so far targeted subjects of mixed agricultural-industrial interest, instead of proper cropping issues. Despite this, the management of industrial crops may not actually be considered backward with respect to "conventional" crops, thanks to the transitive property of many advancements in agronomy.

Areas of potential recovery of the former over the latter still exist, especially in those aspects where a specific work has been tailored to the requirements of large-scale crops, such as in fertilization and irrigation; but the weak points are counterbalanced by the remarkable potential expressed by many industrial crops under several viewpoints: energy efficiency, reduced environmental impact, carbon-sink potential, renewable raw products replacing non-renewable ones, just to mention some of them. Others more are envisaged in the future, in the light of today's research commitment.

References

Aboudrare A, Debaeke P, Bouaziz A, Chekli H (2006) Effects of soil tillage and fallow management on soil water storage and sunflower production in a semi-arid Mediterranean climate. Agric Water Manage in press (doi: 10.1016/j.agwat.2005.12.001)

Adamchuk VI, Hummel JW, Morgan MT, Upadhyaya SK (2004) On-the-go soil sensors for precision agriculture. Comput Electron Agric 44: 71-91

Adamchuk VI, Lund ED, Sethuramasamyraja B, Morgan MT, Dobermann A, Marx DB (2005) Direct measurement of soil chemical properties on-the-go using ion-selective electrodes. Comput Electron Agric 48: 272-294

Al-Kaisi MM, Yin X, Licht MA (2005) Soil carbon and nitrogen changes as influenced by tillage and cropping systems in some Iowa soils. Agric Ecosyst Environ 105: 635-647

Amaducci S, Amaducci MT, Benati R, Venturi G (2000) Crop yield and quality parameters of four annual fibre crops (hemp, kenaf, maize and sorghum) in the North of Italy. Ind Crop Prod 11: 179-186

Amaducci S, Errani M, Venturi G (2002) Plant population effects on fiber hemp morphology and production. J Ind Hemp 7: 33-60.

Amaducci S, Monti A, Venturi G (2004) Non-structural carbohydrates and fibre components in sweet and fibre sorghum as affected by low and normal input techniques. Ind Crop Prod 20: 111-118

Amaducci S (2005) Hemp production in Italy. J Ind Hemp 1: 109-115.

Auernhammer H (2001) Precision farming — the environmental challenge. Comput Electron Agric 30: 31-43

Barbanti L, Grandi S, Vecchi A, Venturi G (2006) Sweet and fibre sorghum (*Sorghum bicolor* (L.) Moench), energy crops in the frame of environmental protection from excessive nitrogen loads. Eur J Agron in press (doi: 10.1016/j.eja.2006.03.001)

Basso B, Ritchie JT, Pierce FJ, Braga RP, Jones JW (2001) Spatial validation of crop models for precision agriculture. Agric Syst 68: 97-112

Bescansa P, Imaz MJ, Virto I, Enrique A, Hoogmoed WB (2006) Soil water retention as affected by tillage and residue management in semiarid Spain. Soil Till Res 87: 19-27

Bhattacharyya R, Prakash V, Kundu S, Gupta HS (2006) Effect of tillage and crop rotations on pore size distribution and soil hydraulic conductivity in sandy clay loam soil of the Indian Himalayas. Soil Till Res 86: 129-140

Bledsoe BL, Bales BM (1992) Bale wrap evaluation. ASAE Paper No. 921573

Botta GF, Jorajuria D, Balbuena R, Ressia M, Ferrero C, Rosato H, Tourn M (2006) Deep tillage and traffic effects on subsoil compaction and sunflower (*Helianthus annus* L.) yields. Soil Till Res in press (doi:10.1016/j.still.2005.12.011)

Chen Y, Tessier S, Irvine B (2004) Drill and crop performances as affected by different drill configurations for no-till seeding. Soil Till Res 77: 147-155

Clifton-Brown JC, Lewandowski I (2000) Water use efficiency and biomass partitioning of three different Miscanthus genotypes with limited and unlimited water supply. Ann Bot 86: 191-200

Coble CG, Egg R (1987) Dry matter losses during hay production and storage of sweet sorghum used for methane production. Biomass 14: 209-217

Condon AG, Richards RA, Rebetzke GJ, Farquhar GD (2004) Breeding for high water-use efficiency. J Exp Bot 55: 2447-2460

Connor DJ, Sadras VO (1992) Physiology of yield expression in sunflower. Field Crop Res 30: 333-389

Consoli S, D'Urso G, Toscano A (2006) Remote sensing to estimate ET-fluxes and the performance of an irrigation district in southern Italy. Agric Water Manage 81: 295-314

Corwin DL, Plant RE (2005) Applications of apparent soil electrical conductivity in precision agriculture. Comput Electron Agric 46: 1-10

Culshaw D, Stokes B (1995) Mechanization of short rotation forestry. Biomass Bioenerg 9, 127-140.

Dam RF, Mehdi BB, Burgess MSE, Madramootoo CA, Mehuys GR, Callum IR (2005) Soil bulk density and crop yield under eleven consecutive years of corn with different tillage and residue practices in a sandy loam soil in central Canada . Soil Till Res 84: 41-53

Damas M, Prados AM, Gómez F, Olivares G (2001) HidroBus® system: fieldbus for integrated management of extensive areas of irrigated land. Microprocess Microsy 25: 177-184

Deen W, Kataki PK (2003) Carbon sequestration in a long-term conventional versus conservation tillage experiment. Soil Till Res 74: 143-150

Di Virgilio N, Monti A, Venturi G (2006) Spatial variability of switchgrass (Panicum virgatum L.) yield as related to soil parameters in a small field. Accepted by Field Crop Res

Duiker SW, Beegle DB (2005) Soil fertility distributions in long-term no-till, chisel/disk and moldboard plow/disk systems. Soil Till Res in press (doi: 10.1016/j.still.2005.04.004)

Ercoli L, Mariotti M, Masoni A, Bonari E (1999) Effect of irrigation and nitrogen fertilization on biomass yield and efficiency of energy use in crop production of Miscanthus. Field Crop Res 63: 3-11

Farré I, Faci JM (2006) Comparative response of maize (*Zea mays* L.) and sorghum (*Sorghum bicolor* L. Moench) to deficit irrigation in a Mediterranean environment. Agric Water Manage in press (doi:10.1016/j.agwat.2005.11.001)

Fismes J, Vong PC, Guckert A, Frossard E (2000) Influence of sulphur on apparent N-use efficiency, yield and quality of oilseed rape (*Brassica napus* L.) grown on a calcareous soil. Eur J Agron 12: 127-141

Flagella Z, Rotunno T, Tarantino E, Di Caterina R, De Caro A (2002) Changes in seed yield and oil fatty acid composition of high oleic sunflower (*Helianthus annuus* L.) hybrids in relation to the sowing date and the water regime. Eur J Agron 17: 221-230

Fortes PS, Platonov AE, Pereira LS (2005) GISAREG—A GIS based irrigation scheduling simulation model to support improved water use. Agric Water Manage 77: 159-179

Frank AB, Berdahl JD, Hanson JD, Liebig MA, Johnson HA (2004) Biomass and Carbon Partitioning in Switchgrass. Crop Sci. 4: 1391-1396

Gajri PR, Gill KS, Chaudhary MR, Singh R (1997) Irrigation of sunflower (*Helianthus annuus*) in relation to tillage and mulching. Agric Water Manage 34: 149-160

Gale F, Tuan F, Lohmar B, Hsu H, Gilmour B (2002) China's food and agriculture: issues for the 21st century. USDA Economic Research Service Agricultural Information Bulletin no. AIB775, Washington, DC

Garten CT, Wüllschleger SD (2000) Soil carbon dynamics beneath switchgrass as indicated by stable isotope analysis. J. Environ. Qual. 29: 645-653

Gherbin P, De Franchi AS, Lupo F (2005) Esperienze sulla propagazione della canna comune (*Arundo donax* L.) (in Italian). In :Giuliani MM, Gatta G (eds) Atti XXXVI Convegno Società Italiana di Agronomia, Foggia, I, pp 262-263

Godwin RJ, Wood GA, Taylor JC, Knight SM, Welsh JP (2003) Precision farming of cereal crops: a review of a six year experiment to develop management guidelines. Biosyst Eng 84: 375-391

Göksoy AT, Demir AO, Turan ZM, Dağüstü N (2004) Responses of sunflower (*Helianthus annuus* L.) to full and limited irrigation at different growth stages. Field Crop Res 87: 167-178

Graeff S, Claupein W (2003) Quantifying nitrogen status of corn (*Zea mays* L.) in the field by reflectance measurements. Eur J Agron 19: 611-618

Hadders G, Olsson R (1997) Harvest of grass for combustion in late sum-mer and in spring. Biomass Bioenerg 3: 171-175

Hamilton-Manns M, Ross CW, Horne DJ, Baker CJ (2002) Subsoil loosening does little to enhance the transition to no-tillage on a structurally degraded soil. Soil Till Res 68: 109-119

Huang M, Shao M, Zhang L, Li Y (2003) Water use efficiency and sustainability of different long-term crop rotation systems in the Loess Plateau of China. Soil Till Res 72: 95-104

Huisman W, Kortleve WJ (1994) Mechanization of crop establishment, harvest, and post harvest conservation of *Miscanthus sinensis* x *Giganteus*. Ind Crops Prod 2: 289-297

Jaggard KW, Stafford J, Lark RM, Fisher S (2000) Are there opportunities for profitable use of precision agriculture in sugar beet crops? In: Proceedings of the 63th IIRB Congress. Interlaken, pp 317-323

Jeuffroy MH, Recous S (1999) Azodyn: a simple model simulating the date of nitrogen deficiency for decision support in wheat fertilization. Eur J Agron 10: 129-144

Jia L, Buerkert A, Chen X, Roemheld V, Zhang F (2004) Low-altitude aerial photography for optimum N fertilization of winter wheat on the North China Plain. Field Crop Res 89: 389-395

Koch HJ, Stockfisch N (2006) Loss of soil organic matter upon ploughing under a loess soil after several years of conservation tillage. Soil Till Res 86: 73-83

Lankford B (2004) Resource-centred thinking in river basins; should we revoke the crop water requirement approach to irrigation planning? Agric Water Manage 68: 33-46

Laureti D, Monotti M, Conti D, Del Pino AM, Pieri S, Ridoni G (2006) Girasole: panorama varietale per il 2006 (in Italian). L'Informatore Agrario 10: 34-39

Lewandowski I, Clifton-Brownb JC, Scurlockc JMO, Huismand W (2000) Miscanthus: European experience with a novel energy crop. Biomass Bioenerg 19: 209-227

Li XY, Gong JD, Gao QZ, Li FR (2001) Incorporation of ridge and furrow method of rainfall harvesting with mulching for crop production under semiarid conditions. Agric Water Manage 50: 173-183

Li XY, Gong JD (2002) Effects of different ridge:furrow ratios and supplemental irrigation on crop production in ridge and furrow rainfall harvesting system with mulches. Agric Water Manage 54: 243-254

Liebig MA, Tanaka DL, Wienhold BJ (2004) Tillage and cropping effects on soil quality indicators in the northern Great Plains. Soil Till Res 78: 131-141

Liebig MA, Johnsons HA, Hanson JD, Frank AB (2005) Soil Carbon under Switchgrass Stands and Cultivated Cropland. Biom. Bioenerg. 28: 347-354

Long SP, Zhu XG, Naidu SL, Ort DR (2006) Can improvement in photosynthesis increase crop yield? Plant Cell Environ 29: 315-330

Ludlow MM (1989) Strategies of response to water stress. In: Kreeb KH, Ritcher H, Hinckley TM (eds) Structural and Functional Responses to Environmental Stresses, SPB Academic Publishing, The Hague, The Netherlands, pp 269-281

Machet JM, Dubrulle P, Louis P (1990) AZOBIL: a computer program for fertilizer N recommendations based on a predictive balance sheet method. In: Scaife A (ed) Proc. 1st Congress of the European Society of Agronomy, Paris, pp 21-22

Malhi SS, Lemke R, Wang ZH, Chhabra BS (2006) Tillage, nitrogen and crop residue effects on crop yield, nutrient uptake, soil quality, and greenhouse gas emissions. Soil Till Res in press (doi: 10.1016/j.still.2005.09.001)

Mastrorilli M, Katerji N, Rana G (1999) Productivity and water use efficiency of sweet sorghum as affected by soil water deficit occurring at different vegetative growth stages. Eur J Agron 11: 207-215

Matson PA, Parton WJ, Power AG, Swift MJ (1997) Agricultural intensification and ecosystem properties. Science 277: 504-509

Metherell AK, Cambardella CA, Parton WJ, Petersen GA, Harding LA, Cole CV (1995) Simulation of soil organic matter dynamics in dryland wheat-fallow cropping systems. In: Lal R, Kimble J, Levine E, Stewart BA (eds) Soil Management and Greenhouse Effect. Adv. Soil Sci., CRC-Lewis Publishers, Boca Raton, pp 259-270

Meynard JM, Doré T, Lucas P (2003) Agronomic approach: cropping systems and plant diseases. C R Biol 326: 37-46

Monti A, Amaducci MT, Pritoni G, Venturi G (2005a) Growth, fructan yield and quality of chicory (*Cichorium intybus* L.) as related to photosynthetic capacity, harvest time and water regime. J Exp Bot 56: 1389-1395

Monti A, Venturi P, Elbersen HW (2001) Evaluation of the establishment of lowland and upland switchgrass (*Panicum virgatum* L.) varieties under different tillage and seedbed conditions in northern Italy. Soil Till Res 63: 75-83

Monti A, Venturi G, Amaducci MT (2002). Comparison among sorghum, kenaf and miscanthus for energy production under different water and nitrogen levels. Riv Agron 36: 213-220

Monti A, Amaducci MT, Venturi G (2005b) Growth response, leaf gas exchange and fructans accumulation of Jerusalem artichoke (*Helianthus tuberosus* L.) as affected by different water regimes. Eur J Agron 23: 136-145

Morari F , Lugato E, Borin E (2004) An integrated non-point source model-GIS system for selecting criteria of best management practices in the Po Valley, North Italy. Agric Ecosyst Environ 102: 247-262

Moser LE (1980) Quality of forages as affected by post-harvest storage and processing. In: Crop Quality, Storage and Utilization, Am Soc Agron, pp 227-260

Murali NS, Secher BJM, Rydahl P, Andreasen FM (1999) Application of information technology in plant protection in Denmark: from vision to reality. Comput Electron Agric 22: 109-115

Murillo JM, Moreno F, Pelegrín F, Fernández J (1998) Responses of sunflower to traditional and conservation tillage under rainfed conditions in southern Spain. Soil Till Res 49: 233-241

Németh K (1982) Electro-ultrafiltration of aqueous soil suspension with simultaneously varying temperature and voltage. Plant Soil 64: 7-23

Oenema O, Kros H, de Vries W (2003) Approaches and uncertainties in nutrient budgets: implications for nutrient management and environmental policies. Eur J Agron 20: 3-16

Oerke EC, Dehne HW (2004) Safeguarding production—losses in major crops and the role of crop protection. Crop Prot 23: 275-285

Olson KR, Lang JM, Ebelhar SA (2005) Soil organic carbon changes after 12 years of no-tillage and tillage of Grantsburg soils in southern Illinois. Soil Till Res 81: 217-225

Pagliai M, Vignozzi N, Pellegrini S (2004) Soil structure and the effect of management practices. Soil Till Res 79: 131-143

Parrish DJ, Wolf DD, Peterson PR, Daniels WL (1997) Switchgrass as a biofuels crop for the upper Southeast: Variety trials and cultural improvements. Final Report (1992-97) for Biofuels Feedstock Development Program, Environmental Sciences Division, Oak Ridge National Laboratory

Passioura J (2006) Increasing crop productivity when water is scarce—from breeding to field management. Agric Water Manage 80: 176-196

Piovanelli C, Gamba C, Brandi G, Simoncini S, Batistoni E (2005) Tillage choices affect biochemical properties in the soil profile. Soil Till Res in press (doi: 10.1016/j.still.2005.08.013)
Rasmussen KJ (1999) Impact of ploughless soil tillage on yield and soil quality: A Scandinavian review. Soil Till Res 53: 3-14
Rees DVH (1982) A discussion of the sources of dry matter loss during the process of haymaking. J Agric Eng Res 27 : 469-479
Rémy JC, Hébert J (1977) Le devenir des engrais azotes dans le sol (in French), CR Acad Agric France 63, pp 700-710
Rémy JC, Viaux P (1982) The use of nitrogen fertilizers in intensive wheat growing in France. In: Symposium on Fertilizers and Intensive Wheat Production in EEC, The Fertilizer Society, pp 67-92
Reyniers M, Vrindts E, De Baerdemaeker J (2006) Comparison of an aerial-based system and an on the ground continuous measuring device to predict yield of winter wheat. Eur J Agron 24: 87-94
Rinaldi M (2001) Application of EPIC model for irrigation scheduling of sunflower in Southern Italy. Agric Water Manage 49: 185-196
Rinaldi M, Losavio N, Flagella Z (2003) Evaluation and application of the OILCROP–SUN model for sunflower in southern Italy. Agric Syst 78: 17-30
Robinson TP, Metternicht G (2005) Comparing the performance of techniques to improve the quality of yield maps. Agric Syst 85: 19-41
Rosenzweig C, Strzepek KM, Major DC, Iglesias A, Yates DN, McCluskey A, Hillel D (2004) Water resources for agriculture in a changing climate: international case studies. Global Environ Chang Part A 14: 345-360
Russell JR, Buxton DR (1985) Storage of large round bales of hay at different moisture concentrations and treated with sodium diacetate and/or covered with plastic. Animal Feed Sci Tech 13: 69-81
Salinas-García JR, Velázquez-García JJ, Gallardo-Valdez M, Díaz-Mederos P, Caballero-Hernández F, Tapia-Vargas LM, Rosales-Robles E (2002) Tillage effects on microbial biomass and nutrient distribution in soils under rain-fed corn production in central-western Mexico. Soil Till Res 66: 143-152
Sanderson MA, Reed RL, Hussey MA, Tischler CR, Read JC, Ocumpaugh WR (1997a) Switchgrass management for biomass energy feedstock in Texas. In: Proc. 18th Int. Grassld. Cong., Winnipeg, Manitoba, and Saskatoon, Sasskatchewan, Canada
Sanderson MA, Egg RP, Wiselogel AE (1997b) Biomass losses during harvest and storage of switchgrass. Biomass Bioenerg 2: 107-114
Scherer HW (2001) Sulphur in crop production — invited paper. Eur J Agron 14: 81-111
Schittenhelm S (1999) Agronomic performance of root chicory, Jerusalem artichoke and sugarbeet in stress and non-stress environment. Crop Sci 39: 1815–1823

Scotford IM, Miller PCH (2005) Applications of spectral reflectance techniques in Northern European cereal production: a review. Biosyst Eng 90: 235-250
Sehy U, Ruser R, Munch JC (2003) Nitrous oxide fluxes from maize fields: relationship to yield, site-specific fertilization, and soil conditions. Agric Ecosyst Environ 99: 97-111
Sepaskhah AR, Kanooni A, Ghasemi MM (2003) Estimating water table contributions to corn and sorghum water use. Agric Water Manage 58: 67-79
Sieling K, Kage H (2006) N balance as an indicator of N leaching in an oilseed rape – winter wheat – winter barley rotation. Agric Ecosyst Environ 115: 261-269
Singh B, Malhi SS (2006) Response of soil physical properties to tillage and residue management on two soils in a cool temperate environment. Soil Till Res 85: 143-153
Somerville C, Briscoe J (2001) Genetic engineering and water. Science 292: 2217
Song G, Li L, Pan G, Zhang Q (2005) Topsoil organic carbon storage of China and its loss by cultivation. Biogeochemistry 74: 47–62
Tan Z, Lal R (2005) Carbon sequestration potential estimates with changes in land use and tillage practice in Ohio, USA. Agric Ecosyst Environ 111: 140-152
Tavakkoli AR, Oweis TY (2004) The role of supplemental irrigation and nitrogen in producing bread wheat in the highlands of Iran. Agric Water Manage 65: 225-236
Thomson AM, Izaurralde RC, Rosenberg NJ, He X (2006) Climate change impacts on agriculture and soil carbon sequestration potential in the Huang-Hai Plain of China. Agric Ecosyst Environ 114: 195-209
Todorovic M, Steduto P (2003) A GIS for irrigation management. Phys Chem Earth Parts A/B/C 28: 163-174
Tolk JA, Howell TA (2003) Water use efficiencies of grain sorghum grown in three USA southern Great Plains soils. Agric Water Manage 59: 97-111
Vamerali T, Bertocco M, Sartori L (2005) Effects of a new wide-sweep opener for no-till planter on seed zone properties and root establishment in maize (*Zea mays*, L.): A comparison with double-disk opener. Soil Till Res in press (doi: 10.1016/j.still.2005.07.011)
Venturi G. (1970) Confronto fra metodi di raccolta della canapa. Nota II. Destinazione cartaria del prodotto. Industria della Carta 1: 8-18.
Venturi G. (2004) The hemp between tradition and innovation. Int. Symposium: Hemp: perspectives for advanced utilization. Bologna, March 5-6, in press
Venturi P, Monti A, Piani I, Venturi G (2004) Evaluation of harvesting and post-harvesting techniques for energy destination of switchgrass. Proc. of the II World Conference on Biomass for Energy, Industry and Climate Protection, Rome, 1, pp 234-236
Vyn TJ, Galic DM, Janovicek KJ (2002) Corn response to potassium placement in conservation tillage. Soil Till Res 67: 159-169
Wang WJ, Dalal RC (2005) Carbon inventory for a cereal cropping system under contrasting tillage, nitrogen fertilisation and stubble management practices. Soil Till Res in press (doi:10.1016/j.still.2005.11.005)

Weiss EA (1983) Oilseed crops. Longman Ltd, pp 660
Wiklicky L (1982) Application of the EUF procedure in sugar beet cultivation. Plant Soil 64: 115-127
Xue Q, Zhu Z, Musick JT, Stewart BA, Dusek DA (2006) Physiological mechanisms contributing to the increased water-use efficiency in winter wheat under deficit irrigation. J Plant Physiol 163: 154-164
Yadava UL (1986) A rapid and non-destructive method to determine chlorophyll in intact leaves. HortScience 21: 1449-1450
Zamuner EC, Picone LI, Echeverria HE (2005) Comparison of phosphorus fertilization diagnostic methods for wheat under no-tillage. Soil Till Res in press (doi: 10.1016/j.still.2005.06.010)

5

Lignin manipulation for fibre improvement

Jennifer Stephens, Claire Halpin

Plant Research Unit, School of Life Sciences, University of Dundee at SCRI, Invergowrie, Dundee DD2 5DA, UK (e-mail: c.halpin@dundee.ac.uk)

1. Introduction

For centuries plant fibres have been used in a number of commercial areas including textiles, construction, paper and pulp, reinforced composites, and as biomass for energy production. These fibres come from a whole host of crops ranging from cotton, jute and flax for textiles; wood crops such as poplar, eucalyptus and conifers for paper and pulp; and cereal crops such as maize, sorghum and barley to provide straw, bedding and animal fodder. In more recent years the popularity of fibre crops in some of these areas has been superseded by synthetic fibres such as those made from plastic or glass. Environmentally, these synthetic fibres are non-renewable and continue to accumulate as sources of pollution. The impact of this pollution has led to a renewed interest in the use of plant fibres as a sustainable commodity for the future.

With the exception of cotton fibres, which are almost pure cellulose, most plant fibres also contain hemicelluloses, pectins and lignins connected together in a complex network. Although each of these components is likely to have an effect on the properties of the plant fibre that is produced, little is known of the basic biological processes underlying the production of most of these polymers. It has been estimated that 15% of the transcribed genome of *Arabidopsis* comprises genes directly involved in cell wall metabolism but few of these genes have been studied in any detail.

P. Ranalli (ed.), Improvement of Crop Plants for Industrial End Uses, 129–153.

Of the different cell wall polymers, the most intensively studied to date has been lignin. Interest in lignin biosynthesis has been driven mainly by the perceived value that improvements in the extractability of lignin would have to the pulp and paper industry. Manipulating genes in the lignin biosynthesis pathway has also been shown to have an impact on the digestibility of certain forage crops. Most of the genes in the lignin biosynthesis pathway (Fig. 5.1) have now been cloned, characterized and manipulated in a range of species including *Arabidopsis,* herbaceous woody angiosperms (tobacco) and trees (poplar and eucalyptus). This available data offers a starting point for further research into fibre crop improvement by focusing on manipulating lignin genes. Although genetic modification (GM) of crop plants offers the greatest potential for rapidly improving fibre traits, research in this area is currently limited due to public concerns over the safety of GM. As an alternative approach, conventional breeding can still benefit from the latest biotechnological tools which are being used to identify candidate genes for marker-assisted breeding.

2. State-of-the-Art and the latest advances of the sector

2.1 Lignin

Lignin is an important component of plant secondary cell walls. It is a complex polymer of phenylpropanoid units that is cross-linked to other components, including cellulose, within the wall. The integration of lignin into the cell wall strengthens and maintains the structure of the cell. Lignin also offers the plant some protection against external threats such as herbivores and pathogens. During papermaking, lignin is difficult to degrade, demanding the use of highly toxic chemicals during its extraction, which go on to pollute the environment. As a component in forage crops, lignin makes it difficult for animals to break down the plant fibre to release the available energy. Much research has been done on manipulating the genes that are involved in lignin biosynthesis in an attempt to make lignin that is more amenable to extraction during pulping (Baucher et al. 2003), and to improve the digestibility of forage crops (Barriere et al. 2003).

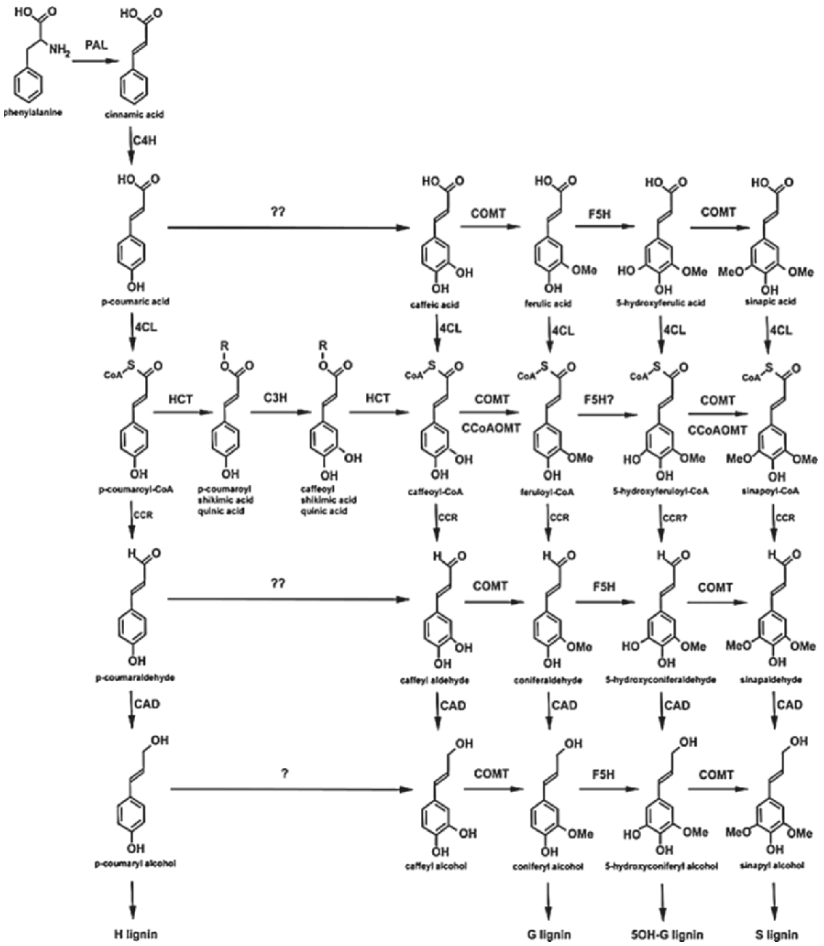


Fig. 5.1. The lignin biosynthesis pathway (adapted from Raes et al. 2003). PAL, Phenylalanine ammonia lyase; C4H, Cinnamate-4-hydroxylase; 4CL, 4-Coumarate:CoA ligase; HCT, Hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyltransferase; C3H, Coumarate-3-hydroxylase; COMT, Caffeate 3-*O*-methyltransferase; CCoAOMT, Caffeoyl CoA 3-*O*-methyltransferase; CCR, Cinnamoyl-CoA reductase; F5H, Ferulate-5-hydroxylase; CAD, Cinnamyl alcohol dehydrogenase.

There are three monomers (monolignols) produced by the lignin pathway that become major components of the lignin polymer - *para*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) monomers (Fig. 5.2). These monomers differ only in their degree of methoxylation. The percentage of each monomer that is incorporated into lignin varies from species to species, and also within plants from cell type to cell type (Campbell and Sederoff 1996). Generally, lignin in gymnosperms is predominantly composed of G units while angiosperm lignin is a mixture of G and S units, with lignin in the grasses additionally containing H units.

The content, composition and structure of lignin within cell walls, as well as the type and frequency of linkages between lignin and other wall polymers, influence the mechanical and industrial properties of plant fibres. A considerable body of research, summarized in the next section, illustrates how the physical and chemical properties of fibres can be modified by manipulating lignin. Most of this work focuses on improving wood for applications in pulping or improving forages as animal feedstocks. However the results of this research are equally relevant to the improvement of other types of fibre crop, irrespective of whether or not they have applications in pulping or animal feed production, since they demonstrate the types and extent of manipulation that is possible without adverse effects on plant health and viability.

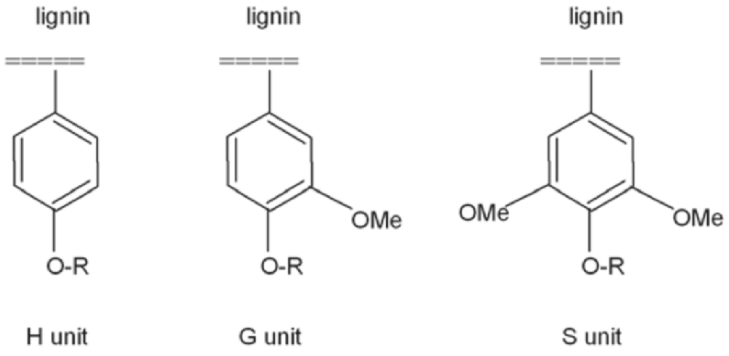


Fig. 5.2. The major monomers incorporated into lignin: *para*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units.

2.2 Manipulation of genes involved in lignin biosynthesis

All of the genes known to be involved in lignin biosynthesis have been targeted for manipulation or mutation in model plants such as *Arabidopsis*, tobacco, or poplar. There are currently ten genes known to be involved in monolignol production. The roles of each of these genes have been characterized following up- or down-regulation of gene activity in transgenic plants, either in a single construct or in combination with other lignin genes. Mutants have also been isolated for some of the lignin genes. A brief explanation of their role in the pathway follows along with results from studies of gene manipulation.

2.2.1. Phenylalanine ammonia lyase (PAL)

PAL is the first enzyme in the pathway and catalyses the deamination of phenylalanine to trans-cinnamic acid (Wanner et al. 1995). This reaction is regulated by a feedback loop that inhibits PAL activity in the presence of high levels of trans-cinnamic acid (Blount et al. 2000). Down-regulating PAL activity in tobacco (Sewalt et al. 1997a) leads to a decrease in the content of G lignin, but does not alter the content of S lignin. Although down-regulating PAL can have a big impact on lignin content, it frequently leads to the production of plants with pleiotrophic detrimental phenotypes making PAL unsuitable as a target for improving fibre qualities while maintaining normal plant growth.

2.2.2. Cinnamic acid 4-hydroxylase (C4H)

Trans-cinnamic acid is hydroxylated by C4H to produce p-coumaric acid. C4H belongs to the family of cytochrome P450-dependent monooxygenases. It has been shown that C4H activity is tightly co-ordinated with PAL activity (Koopmann et al. 1999). Down-regulating activity of C4H in tobacco (Sewalt et al. 1997a) and alfalfa (Reddy et al. 2005) plants results in a reduction in lignin content and a decrease in the S/G ratio. Although the lignin composition in the C4H down-regulated alfalfa plants was not hugely altered, there was a large improvement in digestibility, suggesting that lignin content rather than lignin composition may be a key determinant in improving digestibility.

2.2.3. Coumarate:CoA ligase (4CL)

There are multiple isoforms of 4CL in *Arabidopsis* which catalyze the formation of CoA esters of p-coumaric acid, caffeic acid, ferulic acid, 5-hydroxyferulic acid and sinapic acid (Raes et al. 2003). Down-regulating activity of 4CL in tobacco plants results in reduced lignin content and the plants with the greatest lignin reductions were stunted and had collapsed xylem vessels (Kajita et al. 1997). The S/G ratio in the plants was decreased, although studies in plants other than tobacco report an increased S/G ratio (as reviewed by Boerjan et al. 2003). In poplar, reduced lignin in 4CL-down-regulated plants is apparently compensated for by an increase in cellulose in healthy plants that grow taller than normal (Hu et al. 1999), although the growth effects depend on the promoter used to drive the transgene (Li et al. 2003a).

2.2.4. HydroxycinnamoyI-CoenzymeA Shikimate/Quinate Hydroxycinnamoyl Transferase (HCT)

HCT has recently been identified as acting upstream and downstream of C3H. It catalyzes the conversion of *p*-coumaroyl-CoA to the corresponding shikimic acid or quinic acid esters. It also catalyzes the reverse reaction leading to caffeoyl-CoA. Down-regulation of HCT results in plants with a reduced lignin content, containing less S units, and more H units. *Arabidopsis* plants severely down-regulated in HCT (with ~1% of wild type activity) have almost no S units in the interfascicular fibre cells. These plants have a dwarf phenotype and are sterile (Hoffmann et al. 2004).

2.2.5. Coumarate-3-hydroxylase (C3H)

C3H catalyzes the hydroxylation of shikimic acid and quinic acid esters to caffeoyl units (Schoch et al. 2001). C3H is a P450-dependent monooxygenase. The *Arabidopsis* C3H-defective mutant (*ref*8) has a dwarf phenotype, yielding significantly less lignin than the wild type. *Ref*8 lignin is composed almost exclusively of H units which are only found in trace amounts in the wild type (Franke et al. 2002). A reduction in hemicellulose and lignin was found in C3H down-regulated alfalfa plants (Reddy et al. 2005) which showed evidence of compensatory cellulose accumulation, as previously reported in 4CL down-regulated poplar plants. Although the most severely down-regulated alfalfa plants showed delayed growth, the majority of the plants were phenotypically similar to wild type alfalfa despite having dramatically altered lignin. These alfalfa plants had a massive increase in H units with the biggest increase in digestibility yet found in forage crops. The dramatic changes in lignin structure due to the altered

composition of the polymer are thought to be responsible for the significant digestibility improvement (Ralph et al. 2006).

2.2.6. Caffeic acid 3-O-methyltransferase (COMT)

COMT has been implicated to act at different levels in the pathway (Inoue et al. 1998; Maury et al. 1999). It is currently thought to be predominantly active at the level of the aldehydes and alcohols, leading to S lignin biosynthesis. Down-regulation of COMT in tobacco produces plants with a phenotype indistinguishable from wild type plants. The amount of lignin is not significantly reduced, but the composition of the lignin is altered, containing less S units, and abnormally high amounts of 5-hydroxyguaiacyl (5-OH-G) units (Atanassova et al. 1995; Pinçon et al. 2001a). The incorporation of 5-OH-G units following down-regulation of COMT has also been reported in poplar (Jouanin et al. 2000) and in maize (Lapierre et al. 1988). COMT mutants have been reported in maize (Vignols et al. 1995) and sorghum (Porter et al. 1978). These mutants have a brown midrib phenotype and show reductions in lignin content along with improvements in digestibility (Cherney et al. 1991; Bout and Vermerris 2003). COMT is one of the main candidates for improving digestibility.

2.2.7. Caffeoyl CoA 3-O-methyltransferase (CCoAOMT)

CCoAOMT methylates caffeoyl-CoA to give feruloyl-CoA. Down-regulating activity of CCoAOMT leads to a reduction in lignin content. It has no effect on S units, but shows a reduction in the amount of G units in tobacco (Pinçon et al. 2001a) and alfalfa (Guo et al. 2001a) plants. The alfalfa CCoAOMT down-regulated plants reportedly had a much higher digestibility than wild type plants (Guo et al. 2001b). In poplar, plants suppressed in CCoAOMT made a lignin that was less cross-linked than normal and increased levels of free and bound *p*-hydroxybenzoic acid were detected in the cell walls (Zhong et al. 2000).

2.2.8. Cinnamoyl-CoA reductase (CCR)

CCR is a key enzyme in the pathway converting cinnamoyl-CoA esters to their respective cinnamaldehydes. This is the first committed step in monolignol biosynthesis leading to the production of coniferaldehyde, 5-OH coniferaldehyde and sinapaldehyde. Down-regulating CCR in a variety of species has revealed its important role in regulating lignin content. CCR-suppressed tobacco plants have large reductions in lignin content and changes to lignin structure (Ralph et al. 1998; Piquemal et al. 1998; O'Connell et al. 2002). Similar changes to lignin content and structure

have been identified in transgenic or mutant *Arabidopsis* plants with reduced CCR activity (Jones et al. 2001; Goujon et al. 2003). Many of these CCR-deficient plants do not develop completely normally and have stunted growth, altered leaf morphology and collapsed or irregular xylem vessels. The changes in lignin seem to result in a disorganization and loosening of the secondary walls of fibres and vessels, leading to mechanical weakness (Chabannes et al. 2001a,b; Pinçon et al. 2001b; Goujon et al. 2003).

2.2.9. Ferulate-5-hydroxylase (F5H)

F5H preferentially catalyzes the 5-hydroxylation of coniferaldehyde (Humphreys et al. 1999) leading to the production of the S lignin monomer. F5H is a member of the CYP84 family of P450-dependent monooxygenases. The *Arabidopsis* F5H-deficient mutant (*fah1*; Meyer et al. 1996) exhibits only trace amounts of S units, while over-expression of F5H under the control of the C4H promoter results in a lignin that is almost entirely composed of S units (Marita et al. 1999). Despite the absence of S units, the *fah1* mutant shows no change in cell wall degradability by rumen micro-organisms (Jung et al. 1999). Down-regulation of F5H in alfalfa (Reddy et al. 2005) shows a reduction in S units with no effect on digestibility.

2.2.10. Cinnamyl alcohol dehydrogenase (CAD)

CAD is the final enzyme in the monolignol biosynthesis pathway and reduces *para*-coumaraldehyde, coniferaldehyde and sinapaldehyde into their corresponding alcohols. Down-regulating CAD in various species including tobacco and poplar produces plants with a red-brown colour in the stem wood. This is thought to be due to an increase in the amount of aldehydes incorporated into the lignin in these plants (Higuchi et al. 1994; Baucher et al. 1996). Due to this colour, a number of potential CAD mutants have been identified in maize and sorghum (see Cherney et al. 1991), and recently in rice (Zhang et al. 2006). Experiments on CAD-antisense tobacco plants indicated that the structural and compositional changes of the tobacco lignin changed the mechanical properties of the stem wood, although the possible impact of these changes on plant health and wood processing properties were not directly addressed (Hepworth and Vincent 1998, 1999). However, long-term field trials of poplar trees with reduced CAD activity have shown that tree growth and fitness is normal as are ecological interactions with insects and soil microbes (Pilate et al. 2002) although the trees have significant pulping benefits (discussed below).

2.2.11. Manipulation of multiple monolignol biosynthesis genes

Although some of the genes in the lignin biosynthesis pathway appear to be good candidates for crop improvement, the greatest results may come through manipulating more than one gene at a time. There are a few reports where two or more lignin genes have been targeted simultaneously. Both COMT and CCoAOMT have been simultaneously suppressed in tobacco (Zhong et al. 1998; Pinçon et al. 2001a) and alfalfa (Guo et al. 2001a). In tobacco, the double gene suppression promoted a greater reduction in lignin content than could be achieved by targeting either gene alone. Similarly, tobacco suppressed in both CAD and CCR showed synergistic effects of the two genes in reducing lignin quantity (Chabannes et al. 2001a). Plants suppressed in both genes had lignin decreases of approximately 50% compared to 32% and 12% reductions in plants suppressed in CCR or CAD alone. Simultaneous suppression of COMT and CCR has been achieved in tobacco by crossing plants down regulated in the single genes (Pinçon et al. 2001b). In this case, the effects of CCR suppression on lignin predominated - plants had reduced lignin content and increased S/G ratio but lignin changes typical of COMT suppression were not detected, possibly due to insufficient levels of COMT down-regulation (Pinçon et al. 2001b). Down-regulating CAD and COMT together in tobacco produces plants with a normal phenotype and reduced lignin content, while plants down-regulated in CAD, COMT and CCR together reduces lignin content but produces a stunted phenotype (Abbott et al. 2002). In poplar trees where F5H was up-regulated in tandem with 4CL down-regulation, additive effects were observed compared to transformants where only one of the genes had been manipulated. Lignin composition was altered and lignin content was reduced while cellulose content was increased (Li et al. 2003a). This work illustrates the potential for manipulating different complex traits affecting fibre quality by targeting relatively few genes (Halpin and Boerjan 2003).

2.2.12. Manipulation of monolignol transport and polymerisation

Considerable scope exists for manipulating lignin by targeting processes other than monolignol biosynthesis, such as monolignol transport or polymerization. Indeed these late steps in lignin deposition might be expected to be particularly suitable targets as unintentional effects on connected biochemical pathways could be avoided. Unfortunately, the details of how monolignols are transported and how they become polymerized within plant cell walls is still unclear and several possible mechanisms have been proposed (see Halpin 2004 for review). Monolignols are assumed to be exported from the cell as glucosides, although direct evidence for this is still lacking. In *Arabidopsis*, a number of UDP-glucosyltransferases have been

identified that are capable of glucosylating lignin monomers or their direct precursors (Lim et al. 2001, 2005). Similarly, a gene encoding a coniferin β-glucosidase that is capable of de-glucosylating monolignols has been found in pine (Dharmawardhana et al. 1999). Consistent with a potential role in deglucosylating exported monolignols prior to polymerization, the encoded protein is found in secondary cell walls of developing xylem (Samuels et al. 2002). Manipulating the expression of either the UDP-glucosyltransferases or the β-glucosidases that act on monolignols might be a useful strategy for manipulating lignin content or composition in crops, but, as yet, there are no reports of such work in the literature.

The identity of the enzymes involved in polymerizing lignin monomers is still under debate with peroxidases, laccases, and other phenoloxidases all proposed to play a role. Controversy also exists as to whether lignin polymerization is a random or highly ordered process, perhaps mediated by 'dirigent' proteins (Davin et al. 1997). Several reports describe the effects of manipulating the expression of various peroxidase genes *in planta*. In most cases, modification of anionic peroxidase expression had little effect on lignin deposition in tobacco (Lagrimini et al. 1997a,b) although aspen with reduced activity of a stem-specific anionic peroxidase (prxA3a) had a moderate reduction in lignin content (Li et al. 2003b). Similarly, suppression of laccase activity in poplar had no effect on lignin amount or composition (Ranocha et al. 2002). By contrast, suppression of a cationic peroxidase (TP60) in tobacco apparently reduced lignin content by 40-50% (Blee et al. 2003). However the exact roles of the multiplicity of different peroxidases and laccases present in different plant species, and the possibility of significant functional redundancy between them, makes effective modification of lignin via manipulation of monolignol polymerization an uncertain prospect.

2.2.13. Fibre improvement for pulp and paper making

During pulp production from plant fibres, lignin has to be removed from cellulose in order to produce good-quality paper. However, due to its complex structure, lignin is very difficult to extract, demanding the use of highly toxic chemicals which can pollute the environment. In order to make pulping easier and more environmentally benign, either (a) the lignin content of fibres could be reduced or (b) the extractability of lignin could be improved by modifying its chemical structure. Pulping studies have already been performed on some of the transgenic lignin-modified plants described in the last section and the results demonstrate the potential that exists for manipulating fibres to improve pulping properties.

Transgenic poplar suppressed in CAD activity has been evaluated by chemical pulping analyses after growth in the greenhouse (Baucher et al. 1996; Lapierre et al. 1999) and after two four-year field trials in France and the UK (Pilate et al. 2002). In all cases, the changes to lignin structure in CAD-suppressed plants made delignification easier using the chemical Kraft pulping process. In pulps made from the transgenic lines, the kappa number, a measure of residual lignin in the pulp after cooking, was reduced compared to that of pulp made from wild type plants. Subsequent bleaching of the pulps was also easier and other pulp properties were not adversely affected by the lignin modification. In a pine *cad* mutant, no enhanced delignification was evident after Kraft pulping (MacKay et al. 1999), probably reflecting the differences in lignin structure between gymnosperms and angiosperms. However, pulp produced from the mutant by the soda pulping process had a lower kappa number than wild type. Poplars overexpressing F5H also show significant improvements of Kraft pulping efficiency. Pulps from the F5H-expressing plants had lower kappa numbers and increased brightness compared to wild type (Huntley et al. 2003). The authors estimate that pulp throughput could be increased by 60% and consumption of pulping chemicals decreased by this genetic improvement. Not all lignin modifications are beneficial for pulping, however. Wood from field-grown COMT-down regulated poplars has proved to be more difficult to pulp than wood from wild type trees. The modified lignin was more difficult to extract and the kappa number was higher while pulp brightness after bleaching was lower than that of wild type trees (Pilate et al. 2002).

Pulping performance has also been assessed for transgenic tobacco with various modifications to lignin biosynthesis (O'Connell et al. 2002; Kajita et al. 2002). The Kraft pulp produced from CAD- or CCR-suppressed tobacco had a lower kappa number than pulp made from wild type plants (O'Connell et al. 2002). However, after bleaching, the pulp from the low-CCR plants had reduced brightness. This appeared to be due to a higher content of unextracted chlorophyll (O'Connell et al. 2002). Tobacco suppressed in 4CL has also been shown to be improved for Kraft pulping, having a higher efficiency of delignification, higher pulp yield, and improved subsequent bleaching, than pulp from wild type plants (Kajita et al. 2002).

As well as indicating how trees grown for pulping might be improved, this work also illustrates the potential for using and manipulating less traditional crops for paper production. The tobacco plants analyzed made high quality pulps that could be bleached to high levels of brightness and made into strong papers (O'Connell et al. 2002). This suggests a new opportunity for tobacco to be grown as a pulp crop and illustrates the potential for manipulating other annual crops for improved fibre production for pulp and paper making.

2.2.14. Fibre improvement for the forage industry

Forage crops are the main source of nutrition for ruminating animals. In 2005, over one billion metric tons of forage crops were produced worldwide. The main bulk of this forage material comes from maize and alfalfa production (Fig. 5.3).

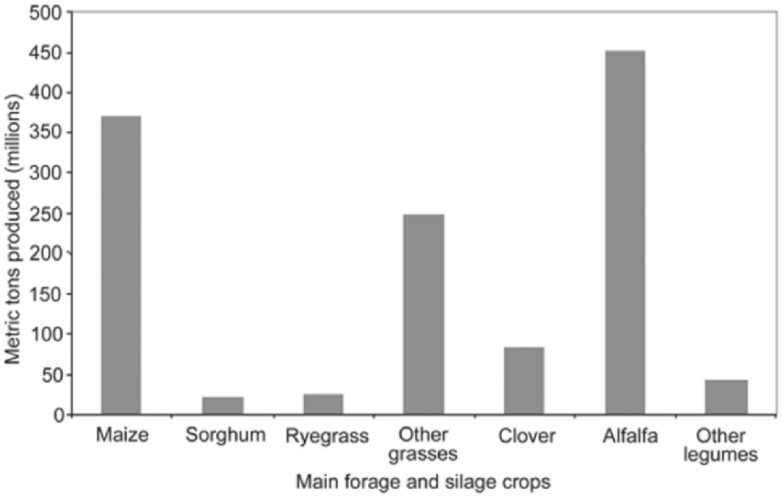


Fig. 5.3. Worldwide production of crop plants grown for forage and silage in 2005 in millions of metric tons (from FAOSTAT data).

The presence of lignin in these crops has a detrimental effect on their digestibility by ruminants (Cherney et al. 1991) due to the resistance of lignin to degradation by micro-organisms. Altering the content and/or composition of lignin by genetic manipulation of genes in the lignin biosynthetic pathway can result in changes to the digestibility of crop plants (see Barriere et al. 2003 for review). Numerous studies have shown that by down-regulating expression of certain genes in the lignin pathway the ratio of S/G monomers can be manipulated. Along with changes in lignin content, even small changes in the S/G ratio can have a significant impact on fibre digestibility as can changes to lignin structure. As already mentioned above, decreases in lignin content in C4H down-regulated alfalfa resulted in a large improvement in digestibility, but the massive structural changes resulting from the incorporation of increased levels of H lignin in C3H-deficient alfalfa had an even greater effect on digestibility. CAD- and COMT-deficient transgenic tobacco and alfalfa have also been shown to have slightly improved digestibility (Bernard-Vailhe et al. 1996, 1998). PAL-suppressed transgenic tobacco or CCR-deficient transgenic *Arabidopsis*

indicated similar improvements to enzymatic digestibility (Sewalt et al. 1997b; Goujon et al. 2003).

Although modifications to digestibility have been demonstrated in GM plants, many of the species concerned are not normally used for forage. A better illustration of how lignin might be manipulated to improve digestibility comes from data on a family of naturally-occurring or chemically-induced lignin mutants, the brown midrib mutants. These mutants have been much used in digestibility studies and have been instrumental in furthering understanding of the basic processes involved during lignification of grass cell walls.

In maize, four naturally occurring brown midrib (*bm*) mutants exist (named *bm*1, *bm*2, *bm*3 and *bm*4), the first of which was discovered in 1924. Brown midrib mutants have now been found in a number of other crop species including sorghum (Bout and Vermerris 2003), millet (Cherney et al. 1988), sudangrass (Fritz et al. 1981), and recently rice (Zhang et al. 2006). These mutants all exhibit a reddish or golden brown pigment in the midrib and stem. Most brown midrib mutants are susceptible to lodging, possibly due to brittleness of the stem caused by a reduction in lignin content. *Bm*1 is the result of a mutation in or near the CAD gene (Halpin et al. 1998) and *bm*3 is the result of a mutation in the COMT gene (Vignols et al. 1995). The genes for *bm*2 and *bm*4 have not yet been identified but it is thought that, if not directly involved in lignin biosynthesis, they could play a related role as transcription factors (Vermerris and Boon 2001) or be involved in transportation of monolignols to the cell wall (Barriere et al. 2004).

Studies of the *bm*1 mutant shows that both the total lignin content and the structure of the lignin polymer are altered (Halpin et al. 1998). These plants have improved lignin extractability, perhaps making them good candidates as novel pulping materials, and have modest improvements in digestibility. The lignin content in *bm*3 plants is reduced by 25-40%, with a reduction in S units. *Bm*3 maize mutants have been grown commercially in the USA for a number of years. Fig. 5.4 shows the combined results from 18 agronomic and dairy cattle feeding trials comparing *bm*3 and regular corn. On average the yield from *bm*3 corn was reduced by 6%, while acid detergent fibre (ADF) and neutral detergent fibre (NDF) were reduced by 3% and 2% (Lauer and Coors 1997). ADF is a measure of the lignin and cellulose components and is used to predict energy content, while NDF is a measure of the total cell wall, used to predict dry matter (DM) intake potential. From this data, calculations were made to predict milk/ton and milk/acre. *Bm*3 corn showed a 4% increase in milk/ton, but a 2% decrease in milk/acre (Lauer and Coors 1997). Clearly, digestibility in *bm*3 is greatly improved compared to wild type maize, although this is at the expense of lower yields. Digestibility in maize decreases after flower-

ing, while yield increases due to grain production. A trade-off therefore exists between digestibility and yield when deciding the optimum harvest time.

Brown midrib mutants have been created in sorghum by chemical mutagenesis. *Bmr*12, *bmr*18 and *bmr*26 are the result of point mutations generating premature stop codons in a COMT gene (Bout and Vermerris 2003). The *bmr* mutants are allelic and show similar characteristics to maize *bm*3 but are distinct in some respects, e.g. *bmr*12 flowers 7 days early (Pedersen et al. 2005).

Further work is needed to engineer or select plants with the increased digestibility found in *bm*3 mutants, while maintaining yield and resistance to lodging. The identification of COMT alleles showing a less dramatic effect than *bm*3 may efficiently improve digestibility without effecting these changes (Barriere et al. 2003).

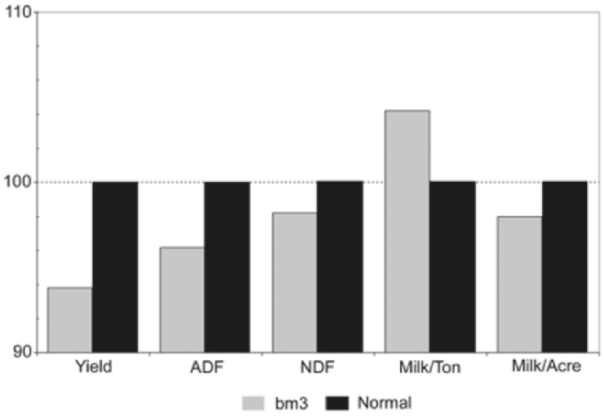


Fig. 5.4. Average yield, ADF, NDF, Milk/Ton and Milk/Acre of *bm3* corn mutants relative to normal corn (100%) compiled from 18 trials (Lauer and Coors 1997).

2.2.15. Crop improvement for textiles and other markets

Besides their use in papermaking and as components of animal forage, one of the biggest markets for natural fibres is still the textile industry. However, an increasing range of new markets are becoming more important, including potential for use of plant fibres in biocomposites for car manufacturing, construction and other industries, and as raw materials for energy production either as biomass for burning or for conversion into biofuels. These emerging commercial opportunities are fuelling new research

initiatives into the 'tailoring' of plant cell walls for different applications but, as yet, few results of such work have been published.

Cotton production dominates the textile market; in 2005, 66 million tons of cotton seed and 23 million tons of cotton fibre were produced worldwide. Cotton fibres, being mostly cellulose, are not targets for lignin manipulation. Instead, major targets in cotton are to improve the yield and produce longer, more uniform fibres to improve the quality of materials for clothing and furnishings. Nevertheless cotton is an example of a genetically modified fibre crop that is already widely deployed commercially, offering hope for growing acceptance of the production of transgenic fibre crops in the future. It is estimated that 28% of the global cotton planted in 2005 was genetically modified (James 2005). Most GM cotton contained an insecticidal *Bt* gene from *Bacillus thuringiensis* which protects the plants from bollworm, an important pest on cotton. However, a significant proportion (37%) of the global GM cotton additionally contained a herbicide-tolerance gene, along with the *Bt* gene, illustrating the state-of-the-art in engineering 'stacked' GM traits into crops and the potential for future manipulation of complex traits, such as fibre quality, with multiple transgenes.

Besides cotton, there are other fibre crops which are of interest to the textile industry (Table 5.1). Of these crops, jute is the most widely grown and has a greater yield per hectare than cotton. The biggest producer of jute is India where 2.8 million tons were produced last year. Jute is used to make lower quality products such as heavy duty clothing, home furnishings and carpets. Jute is 100% biodegradable and has a high tensile strength and a strong resistance to heat and fire. Improvements in the quality of jute fibres would include making the fibres finer so that they would be suitable for use in higher quality products. A jute mutant, *d*eficient *l*ignified *p*hloem *f*ibre (*dlpf*), was recently reported. It contains 50% less lignin, and has a greater amount of cellulose than normal (Sengupta and Palit 2004). This mutant has low levels of PAL activity and has a wavy, undulated, stem phenotype. Although shorter than wild type jute, most of the tissues other than the phloem fibres develop normally. The phloem fibres in the mutant are reduced and are present as single fibres rather than in bundles as they are found in the wild type. The tensile strength of the fibres was not significantly affected even though lignin was greatly reduced. The separateness of the fibres without loss of tensile strength is a trait that could be of interest to textile producers since the fibres have to be mechanically separated from bundles during their preparation for textile production. This is the first lignin mutant to be found in jute, and it shows that there is potential for improving jute by manipulating genes on the lignin pathway.

Table 5.1. Worldwide production, area harvested and yield of crops suitable for use as textiles in 2005 (from FAOSTAT data).

Crop	Production Metric tons $x10^5$	Area Harvested Hectares $x10^3$	Yield Hectograms per hectare $x10^3$
Kapok fruit	3.93	208.00	18.89
Seed cotton	666.66	35217.38	18.93
Cotton lint	232.64	66665.98	3.49
Flax fibre and tow	7.72	501.25	15.40
Hemp fibre and tow	0.67	52.44	12.87
Jute	28.62	1351.91	21.17
Jute-like fibres	3.88	296.20	13.11
Ramie	2.50	131.66	18.95
Sisal	3.28	384.82	8.51
Agave fibres nes	0.58	53.20	10.89
Abaca (Manila hemp)	1.01	147.92	6.83
Fibre crops nes	2.79	89.93	31.02

Production, area harvested and yield of the main crops grown worldwide in 2005 which are suitable for the use in textile making (FAOSTAT data, 2005)

Other crops, such as hemp, are in much lower production although they have the advantages that they can be grown in more moderate climates than cotton, and need reduced inputs in order to achieve high yields (Ebskamp 2002). Along with flax, hemp is one of the most widely grown natural fibre crops in Europe. While its predominant use is in the clothing and home textile industries, hemp has a very wide range of potential and novel applications and is also used for paper, building materials, toiletries, composts, fuel and foods. To make textiles, lignin and pectins have to be removed from the fibre (Wang et al. 2003) and decreasing the lignin content could therefore reduce processing costs and improve the softness of the fibre. It has been reported that improving the fineness of fibre would widen its application in clothing and industrial textiles and that this could be done by breeding hemp varieties containing smaller fibre diameters (Ranalli and Venturi 2004). This reduction in diameters might also potentially be achieved through reducing lignin by manipulating lignin biosynthetic genes.

3. Future and expected developments

Future directed improvement of fibre crops, either by genetic manipulation or conventional breeding, requires increased research effort into identifying the genes involved in all aspects of cell wall biosynthesis. The ongoing development of a variety of genomics tools, following the sequencing of the *Arabidopsis*, rice and poplar genomes, is enabling increasingly rapid identification and functional characterisation of previously 'unknown' genes. Technologies such as DNA microarrays which allow global and parallel analysis of the gene expression profile of an organism or tissue under different conditions are providing vast amounts of information. Transcript profiling in plants is already being used to investigate a wide range of developmental processes, including secondary cell wall deposition. Even microarrays that cover only a small portion of a genome can yield useful data. For example, xylem microarrays have been used to investigate gene expression in distinct development zones within the wood-forming tissues of hybrid aspen (Hertzberg et al. 2001). This analysis revealed the strict transcriptional regulation that must underlie the observed stage-specific expression of different cell wall biosynthesis genes. Similar approaches are currently being used to investigate secondary cell wall formation in fibre crops such as flax and hemp (Ebskamp 2002). Identification of the genes underlying fibre development and cell wall biosynthesis will enable increasingly sophisticated GM strategies to manipulate the expression of combinations of those genes in order to develop 'designer' fibres, improved for conventional and novel applications in the future. However, even without resorting to GM strategies, the identification of increasing numbers of genes affecting fibre quality will enable more rapid breeding improvement through marker assisted selection.

One of the greatest potential benefits of gene expression studies will be the possibility of identifying candidate genes by linking gene expression data to fibre quality QTLs (quantitative trait loci). The development of QTLs, along with comprehensive molecular markers and linkage maps, has enormous potential for marker-assisted breeding (see Collard et al. 2005 for review), enabling breeders to increase the precision of selection and more quickly advance the rate of improvements in crop plants. QTLs have been mapped for a range of agronomically important traits in many plant species. Several QTLs influencing wood properties have already been mapped in tree species used for pulp production, and forage quality QTLs have been identified in several crop species. However, few studies have yet identified the specific genes that underlie these QTLs, although progress is beginning to be made by simply mapping the few genes (such as lignin biosynthetic genes) that have already been shown to affect wood properties in order to see whether they co-locate with mapped QTLs. For

instance, in eucalyptus, specific single nucleotide polymorphisms (SNP) that are significantly associated with microfibril angle, have recently been found in a CCR gene (Thumma et al. 2005). Similarly, in maize, a CCoAOMT gene has been shown to co-localize with a QTL for cell wall digestibility and lignin content (Guillet-Claude et al. 2004). The identification of candidate genes that co-localize with QTLs will enable the development of improved early-selection markers since the linkage between conventional markers and genes can be lost during recombination. Future research will increasingly combine data from QTL analysis of phenotype, genotype and expression levels from microarrays (eQTLs) in genetical genomics strategies (Kirst et al. 2004).

4. Conclusion

Modern, marker-assisted plant breeding, and genetic modification technologies have the potential to improve plant fibres for a variety of conventional and novel end uses. To exploit this opportunity to the full, much research is still needed to identify and characterise the genes involved in fibre cell wall biosynthesis. Using genomic and post-genomic technologies, including transcript profiling, the identification of these genes should proceed at an increasing pace. Verification of the functional role of those genes by isolating the corresponding mutants or by genetic manipulation will also yield the critical information on how the activity of specific genes affects fibre properties. This information can then be applied for fibre improvement. This strategy is already exemplified by the huge body of work over the past decade into the lignin biosynthesis pathway. To date, most, perhaps all, of the genes involved in lignin biosynthesis have been identified and functionally characterized. Mutants and transgenics where expression of those genes has been altered have indicated how fibres can be improved for applications such as pulping and forage use. Modified lignin trees, genetically manipulated for improved pulping, have already been released in controlled field trials, and similar maize mutants with improved digestibility have been marketed in the USA and elsewhere for several years. This work indicates the real opportunities for manipulating lignin and other cell wall polymers for fibre improvement. Continuous innovation in fibre crop breeding either via GM approaches or marker assisted selection, is not only desirable, but is necessary if we are to meet the predicted increased future demand for sustainable, environmentally-friendly, production of natural fibres.

References

Abbott JC, Barakate A, Pinçon G, Legrand M, Lapierre C, Mila I, Schuch W, Halpin C (2002) Simultaneous suppression of multiple genes by single transgenes. Down-regulation of three unrelated lignin biosynthetic genes in tobacco. Plant Physiol. 128:844-853

Atanassova R, Favet N, Martz F, Chabbert B, Tollier MT, Monties B, Fritig B, Legrand M (1995) Altered lignin composition in transgenic tobacco expressing O-methyltransferase sequences in sense and antisense orientation. Plant J. 8:465-477

Barriere Y, Guillet C, Goffner D, Pichon M (2003) Genetic variation and breeding strategies for improved cell wall digestibility in annual forage crops. A review. Anim. Res. 52:193-228

Barriere Y, Ralph J, Mechin V, Guillaumie S, Grabber JH, Argillier O, Chabbert B, Lapierre C (2004) Genetic and molecular basis of grass cell wall biosynthesis and degradability. II. Lessons from brown-midrib mutants. C.R. Biol. 327:847-860

Baucher M, Chabbert B, Pilate G, Van Doorsselaere J, Tollier MT, Petit-Conil M, Cornu D, Monties B, Van Montagu M, Inze D, Jouanin L, Boerjan W (1996) Red xylem and higher lignin extractability by down-regulating a cinnamyl alcohol dehydrogenase in poplar. Plant Physiol. 112:1479-1490

Baucher M, Petit-Conil M, Boerjan W (2003) Lignin: genetic engineering and impact on pulping. Crit. Rev. Biochem. Mol. 38:305-350

Bernard-Vailhe MA, Migne C, Cornu A, Maillot MP, Grenet E, Besle JM, Atanassova R, Martz F, Legrand M (1996) Effect of modification of the *O*-methyltransferase activity on cell wall composition, ultrastructure and degradability of transgenic tobacco. J. Sci. Food Agric. 72:385–391

Bernard-Vailhe MA, Besle JM, Maillot MP, Cornu A, Halpin C, Knight M. (1998). Effect of down-regulation of cinnamyl alcohol dehydrogenase on cell wall composition and on degradability of tobacco stems. J. Sci. Food Agric. 76:505-514

Blee KA, Choi JW, O'Connell AP, Schuch W, Lewis NG, Bolwell GP (2003) A lignin-specific peroxidase in tobacco whose antisense suppression leads to vascular tissue modification. Phytochem. 64:163-176

Blount JW, Korth KL, Masoud SA, Rasmussen S, Lamb C, Dixon RA (2000) Altering expression of cinnamic acid 4-hydroxylase in transgenic plants provides evidence for a feedback loop at the entry point into the phenylpropanoid pathway. Plant Physiol. 122:107-116

Boerjan W, Ralph J, Baucher M (2003) Lignin Biosynthesis. Annu. Rev. Plant Biol. 54:519-546

Bout S, Vermerris W (2003) A candidate-gene approach to clone the sorghum brown midrib gene caffeic acid O-methyltransferase. Mol. Genet. Genomics 269:205-214

Campbell MM, Sederoff RR (1996) Variation in lignin content and composition. Mechanisms of control and implications for the genetic improvement of plants. Plant Physiol. 110:3-13

Chabannes M, Barakate A, Lapierre C, Marita JM, Ralph J, Pean M, Danoun S, Halpin C, Grima-Pettenati J, Boudet AM (2001a) Strong decrease in lignin content without significant alteration of plant development is induced by simultaneous down-regulation of cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) in tobacco plants. Plant J. 28:257-270

Chabannes M, Ruel K, Yoshinaga A, Chabbert B, Jauneau A, Joseleau JP, Boudet AM (2001b) In situ analysis of lignins in transgenic tobacco reveals a differential impact of individual transformations on the spatial patterns of lignin deposition at the cellular and subcellular levels. Plant J. 28:271-282

Cherney JH, Axtell JD, Hassen MM, Anliker KS (1988) Forage quality characterization of a chemically induced brown-midrib mutant in pearl millet. Crop Sci. 28:783-787

Cherney JH, Cherney DJR, Akin DE, Axtell JD (1991) Potential of brown-midrib, low lignin mutants for improving forage quality. Adv. Agron. 46:157-198

Collard BCY, Jahufer MZZ, Brouwer JB, Pang ECK (2005) An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: The basic concepts. Euphytica 142:169-196

Davin LB, Wang HB, Crowell AL, Bedgar DL, Martin DM, Sarkanen S, Lewis NG (1997). Stereoselective bimolecular phenoxy radical coupling by an auxiliary (dirigent) protein without an active center. Science 275:362-366

Dharmawardhana DP, Ellis BE, Carlson JE (1999) cDNA cloning and heterologous expression of coniferin beta-glucosidase. Plant Mol. Biol. 40:365-372

Ebskamp MJM (2002) Engineering flax and hemp for an alternative to cotton. Trends Biotechnol. 20:229-230

Franke R, Hemm MR, Denault JW, Ruegger MO, Humphreys JM, Chapple C (2002) Changes in secondary metabolism and deposition of an unusual lignin in the *ref*8 mutant of *Arabidopsis*. Plant J. 30:47-59

Fritz JO, Cantrell RP, Lechtenberg VL, Axtell JD, Hertel JM (1981) Brown midrib mutants in sudangrass and grain sorghum. Crop Sci. 21:706-709

Goujon T, Ferret V, Mila I, Pollet B, Ruel K, Burlat V, Joseleau JP, Barriere Y, Lapierre C, Jouanin L (2003) Down-regulation of the AtCCR1 gene in *Arabidopsis thaliana*: effects on phenotype, lignins and cell wall degradability. Plants 217:218-228

Guillet-Claude C, Birolleau-Touchard C, Manicacci D, Fourmann M, Barraud S, Carret V, Martinant JP, Barriere Y (2004) Genetic diversity associated with variation in silage corn digestibility for three O-methyltransferase genes involved in lignin biosynthesis. Theor. Appl. Genet. 110:126-135

Guo D, Chen F, Inoue K, Blount JW, Dixon RA (2001a) Downregulation of caffeic acid 3-O-methyltransferase and caffeoyl CoA 3-O-methyltransferase in transgenic alfalfa: impact on lignin structure and implications for the biosynthesis of G and S lignin. Plant Cell 13:73-88

Guo D, Chen F, Wheeler J, Winder J, Selman S, Peterson M, Dixon RA (2001b) Improvement of in-rumen digestibility of alfalfa forage by genetic manipulation of lignin O-methyltransferases. Transgenic Res. 10:457-464

Halpin C (2004) Investigating and manipulating lignin biosynthesis in the post-genomic era. Adv. Bot. Res. 41:63-106

Halpin C, Holt K, Chojecki J, Oliver D, Chabbert B, Monties B, Edwards K, Barakate A, Foxon GA (1998) Brown-midrib maize (bm1) – a mutation affecting the cinnamyl alcohol dehydrogenase gene. Plant J. 14:545-553

Halpin C, Boerjan W (2003) Stacking Transgenes in Forest Trees. Trends Plant Sci. 8, 363-365

Hepworth DG, Vincent JFV (1998) The mechanical properties of xylem tissue from tobacco plants (*Nicotiana tabacum* 'Samsun'). Ann. Bot. 81: 751-759

Hepworth DG, Vincent JFV (1999) The growth response of the stems of genetically modified tobacco plants (*Nicotiana tabacum* 'Samsun') to flexural stimulation. Ann. Bot. 83: 39-43

Hertzberg M, Aspeborg H, Schrader J, Andersson A, Erlandsson R, Blomqvist K, Bhalerao R, Uhlen M, Teeri TT, Lundeberg J, Sundberg B, Nilsson P, Sandberg G (2001) A transcriptional roadmap to wood formation. Proc. Natl. Acad. Sci. U.S.A. 98:14732-14737

Higuchi T, Ito T, Umezawa T, Hibino T, Shibata D (1994) Red-brown color of lignified tissues of transgenic plants with antisense CAD gene: Wine-red lignin from coniferyl aldehyde. J. Biotechnol. 37:151-158

Hoffmann L, Besseau S, Geoffroy P, Ritzenthaler C, Meyer D, Lapierre C, Pollet B, Legrand M (2004) Silencing of Hydroxycinnamoyl-Coenzyme A Shikimate/Quinate Hydroxycinnamoyltransferase affects phenylpropanoid biosynthesis. Plant Cell 16:1446-1465

Hu WJ, Harding SA, Lung J, Popko JL, Ralph J, Stokke DD, Tsai CJ, Chian VL (1999) Repression of lignin biosynthesis promotes cellulose accumulation and growth in transgenic trees Nat. Biotechnol. 17:808-812

Humphreys JM, Hemm MR, Chapple C (1999) New routes for lignin biosynthesis defined by biochemical characterization of recombinant ferulate 5-hydroxylase, a multifunctional cytochrome P450-dependent monooxygenase. Proc. Natl. Acad. Sci. U.S.A. 96:10045-10050

Huntley SK, Ellis D, Gilbert M, Chapple C, Mansfield SD (2003) Significant increases in pulping efficiency in C4H-F5H-transformed poplars: improved chemical savings and reduced environmental toxins. J. Agr. Food Chem. 51:6178-6183

Inoue K, Sewalt VJH, Balance GM, Ni W, Sturzer C, Dixon RA (1998) Developmental expression and substrate specificities of alfalfa caffeic acid 3-O-methyltransferase and caffeoyl coenzyme A 3-O-methyltransferase in relation to lignification. Plant Physiol. 117:761-770

James C 2005 Global Status of Commercialized Biotech/GM Crops: 2005. ISAAA Briefs No. 34. ISAAA: Ithaca, NY

Jones L, Ennos AR, Turner SR (2001) Cloning and characterization of irregular xylem4 (irx4): a severely lignin-deficient mutant of Arabidopsis. Plant J. 26:205-216

Jouanin L, Goujon T, De Nadai V, Martin MT, Mila I, Vallet C, Pollet B, Yoshinaga A, Chabbert B, Petit-Conil M, Lapierre C (2000) Lignification in transgenic poplars with extremely reduced caffeic acid O-methyltransferase activity. Plant Physiol. 123:1363-1373

Jung HJG, Ni W, Chapple CCS, Meyer K (1999) Impact of lignin composition on cell-wall degradability in an *Arabidopsis* mutant. J. Sci. Food Agr. 79:922-928

Kajita S, Hishiyama S, Tomimura Y, Katayama Y, Omori S (1997) Structural characterization of modified lignin in transgenic tobacco plants in which the activity of 4-Coumarate:Coenzyme A Ligase is depressed. Plant Physiol. 114:871-879

Kajita S, Ishifuji M, Ougiya H, Hara S, Kawabata H, Morohoshi N, Katayama Y (2002) Improvement in pulping and bleaching properties of xylem from transgenic tobacco plants. J. Sci. Food Agric. 82:1216-1223

Kirst M, Myburg AA, De Leon JPG, Kirst ME, Scott J, Sederoff R (2004) Coordinated genetic regulation of growth and lignin revealed by quantitative trait locus analysis of cDNA microarray data in an interspecific backcross of Eucalyptus. Plant Physiol. 135:2368-2378

Koopmann E, Logemann E, Hahlbrock K (1999) Regulation and functional expression of cinnamate 4-hydroxylase from parsley. Plant Physiol. 119:49-55

Lagrimini LM, Gingas V, Finger F, Rothstein S, Liu TTY (1997a) Characterization of antisense transformed plants deficient in the tobacco anionic peroxidase. Plant Physiol. 114:1187-1196

Lagrimini LM, Joly RJ, Dunlap JR, Liu TTY (1997b) The consequence of peroxidase overexpression in transgenic plants on root growth and development. Plant Mol. Biol. 33: 887-895

Lapierre C, Tollier MT, Monties B (1988) Occurrence of additional monomeric units in the lignins from internodes of a brown-midrib mutant of maize BM3. CR. Acad. Sci. III-Vie 307:723-728

Lapierre C, Pollet B, Petit-Conil M, Toval G, Romero J, Pilate G, Leple JC, Boer Jan W, Ferret V, De Nadai V, Jouanin L (1999) Structural alterations of lignins in transgenic poplars with depressed cinnamyl alcohol dehydrogenase or caffeic acid O-methyltransferase activity have an opposite impact on the efficiency of industrial kraft pulping. Plant Physiol. 119:153-163

Lauer J, Coors J (1997) Brown midrib corn. Wisconsin Crop Manager 4:16-18

Li L, Zhou Y, Cheng X, Sun J, Marita JM, Ralph J, Chiang V (2003a) Combinatorial modification of multiple lignin traits in trees through multigene cotransformation. Proc. Natl. Acad. Sci. U.S.A. 100:4939-4944

Li YH, Kajita S, Kawai S, Katayama Y, Morohoshi N (2003b) Down-regulation of an anionic peroxidase in transgenic aspen and its effect on lignin characteristics. J. Plant Res. 116:175-182

Lim EK, Li Y, Parr A, Jackson R, Ashford DA, Bowles DJ (2001). Identification of glucosyltransferase genes involved in sinapate metabolism and lignin synthesis in *Arabidopsis*. J. Biol. Chem. 276: 4344-4349

Lim EK, Jackson RG, Bowles DJ (2005) Identification and characterisation of *Arabidopsis* glycosyltransferases capable of glucosylating coniferyl aldehyde and sinapyl aldehyde. FEBS Lett. 579: 2802-2806

MacKay J, Presnell T, Jameel H, Taneda H, O'Malley D, Sederoff R (1999) Modified lignin and delignification with a CAD-deficient loblolly pine. Holzforschung 53:403-410

Marita JM, Ralph J, Hatfield RD, Chapple C (1999) NMR characterization of lignins in *Arabidopsis* altered in the activity of ferulate 5-hydroxylase. Proc. Natl. Acad Sci. U.S.A. 96:12328-12332

Maury S, Geoffroy P, Legrand M (1999) Tobacco O-methyltransferases involved in phenylpropanoid metabolism. The different caffeoyl-coenzyme A/5-hydroxyferuloyl-coenzyme A 3/5-O-methyltransferase and caffeic acid/5-hydroxyferulic acid 3/5-O-methyltransferase classes have distinct substrate specificities and expression patterns. Plant Physiol. 121:215-223

Meyer K, Cusumano JC, Somerville C, Chapple CS (1996) Ferulate-5-hydroxylase from *Arabidopsis* thaliana defines a new family of cytochrome P450-dependent monooxygenases. Proc. Natl. Acad. Sci. U.S.A. 93:6869-6874

O'Connell A, Holt K, Piquemal J, Grima-Pettenati J, Boudet A, Pollet B, Lapierre C, Petit-Conil M, Schuch W, Halpin C (2002) Improved paper pulp from plants with suppressed cinnamoyl-CoA reductase or cinnamyl alcohol dehydrogenase. Transgenic Res. 11:495-503

Pedersen JF, Vogel KP, Funnell DL (2005) Impact of reduced lignin on plant fitness. Crop Sci. 45:812-819

Pilate G, Guiney E, Holt K, Petit-Conil M, Lapierre C, Leplé JC, Pollet B, Mila I, Webster EA, Marstorp HG, Hopkins DW, Jouanin L, Boerjan W, Shuch W, Cornu D, Halpin C (2002) Field and pulping performances of transgenic trees with altered lignification. Nat. Biotechnol. 20:606-612

Pinçon G, Maury S, Hoffmann L, Geoffroy P, Lapierre C, Pollet B, Legrand M (2001a) Repression of O-methyltransferase genes in transgenic tobacco affects lignin synthesis and plant growth. Phytochemistry 57:1167-1176

Pinçon G, Chabannes M, Lapierre C, Pollet B, Ruel K, Joseleau JP, Boudet AM, Legrand M (2001b) Simultaneous down-regulation of caffeic/5-hydroxy ferulic acid-O-methyltransferase I and cinnamoyl-coenzyme A reductase in the progeny from a cross between tobacco lines homozygous for each transgene. Consequences for plant development and lignin synthesis. Plant Physiol. 126:145-155

Piquemal J, Lapierre C, Myton K, O'Connell A, Schuch W, Grima-Pettenati J, Boudet AM (1998) Down-regulation of Cinnamoyl-CoA Reductase induces significant changes of lignin profiles in transgenic tobacco plants. Plant J. 13:71-83

Porter KS, Axtell JD, Lechtenberg VL, Colenbrander VF (1978) Phenotype, fiber composition, and in vitro dry matter disappearance of chemically induced brown midrib (bmr) mutants of sorghum. Crop Sci. 18:205-208

Raes J, Rohde A, Christensen JH, Van de Peer Y, Boerjan W (2003) Genome-wide characterization of the lignification toolbox in *Arabidopsis*. Plant Physiol. 133:1051-1071

Ralph J, Hatfield RD, Piquemal J, Yahiaoui N, Pean M, Lapierre C, Boudet AM (1998) NMR characterization of altered lignins extracted from tobacco plants down-regulated for lignification enzymes cinnamyl-alcohol dehydrogenase and cinnamoyl-CoA reductase. Proc. Natl. Acad. Sci. U.S.A. 95:12803-12808

Ralph J, Akiyama T, Kim H, Lu FC, Schatz PF, Marita JM, Ralph SA, Reddy MSS, Chen F, Dixon RA (2006) Effects of coumarate 3-hydroxylase down-regulation on lignin structure. J. Biol. Chem. 281:8843-8853

Ranalli P, Venturi G (2004) Hemp as a raw material for industrial applications. Euphytica 140:1-6

Ranocha P, Chabannes M, Chamayou S, Danoun S, Jauneau A, Boudet AM, Goffner D (2002) Laccase down-regulation causes alterations in phenolic metabolism and cell wall structure in poplar. Plant Physiol. 129:145-155

Reddy MSS, Chen F, Shadle G, Jackson L, Aljoe H, Dixon RA (2005) Targeted down-regulation of cytochrome P450 enzymes for forage quality improvement in alfalfa (*Medicago sativa* L.) Proc. Natl. Acad. Sci. U.S.A. 102:16573-16578

Samuels AL, Rensing KH, Douglas CJ, Mansfield SD, Dharmawardhana DP, Ellis BE (2002) Cellular machinery of wood production: differentiation of secondary xylem in *Pinus contorta* var. *latifolia*. Planta 216:72-82

Schoch G, Goepfert S, Morant M, Hehn A, Meyer D, Ullmann P, Werck-Reichhart D (2001) CYP98A3 from *Arabidopsis thaliana* is a 3'-hydroxylase of phenolic esters, a missing link in the phenylpropanoid pathway. J. Biol. Chem. 276:36566-36574

Sengupta G, Palit P (2004) Characterization of a lignified secondary phloem fibre-deficient mutant of jute (Corchorus capsularis). Ann. Bot.-London 93:211-220

Sewalt VJH, Ni W, Blount JW, Jung HG, Masoud SA, Howles PA, Lamb C, Dixon RA (1997a) Reduced lignin content and altered lignin composition in transgenic tobacco down-regulated in expression of L-phenylalanine ammonia-lyase or cinnamate 4-hydroxylase. Plant Physiol. 115:41-50

Sewalt VJH, Ni WT, Jung HG, Dixon RA (1997b) Lignin impact on fiber degradation: Increased enzymatic digestibility of genetically engineered tobacco (*Nicotiana tabacum*) stems reduced in lignin content J. Agric. Food Chem. 45:1977-1983

Thumma BR, Nolan MF, Evans R, Moran GF (2005) Polymorphisms in Cinnamoyl CoA Reductase (CCR) are associated with variation in microfibril angle in *Eucalyptus* spp. Genetics 171:1257-1265

Vermerris W, Boon JJ (2001) Tissue-specific patterns of lignification are disturbed in the brown midrib2 mutant of maize (*Zea mays* L.) J. Agr. Food Chem. 49:721-728

Vignols F, Rigau J, Torres MA, Capellades M, Puigdomenech P (1995) The brown midrib3 (bm3) mutation in maize occurs in the gene encoding caffeic acid O-methyltransferase. Plant Cell 7:407-416

Wang HM, Postle R, Kessler RW, Kessler W (2003) Removing pectin and lignin during chemical processing of hemp for textile applications. Text. Res. J. Aug 2003

Wanner LA, Li G, Ware D, Somssich IE, Davis KR (1995) The phenylalanine ammonia-lyase gene family in *Arabidopsis thaliana*. Plant Mol. Biol. 27:327-338

Zhang K, Qian Q, Huang Z, Wang Y, Li M, Hong L, Zeng D, Gu M, Chu C, Cheng Z (2006) Gold hull and internode2 encodes a primarily multifunctional cinnamyl alcohol dehydrogenase in rice. Plant Physiol. 140:972-983

Zhong RQ, Morrison WH, Negrel J, Ye ZH (1998) Dual methylation pathways in lignin biosynthesis. Plant Cell 10:2033-2045

Zhong RQ, Morrison WH, Himmelsbach DS, Poole FL, Ye ZH (2000) Essential role of caffeoyl coenzyme A O-methyltransferase in lignin biosynthesis in woody poplar plants. Plant Physiol. 124:563-577

6

Improvement of fibre and composites for new markets

Marcel Toonen[1], Michel Ebskamp[2], Robert Kohler[3]

[1]Plant Research International, Wageningen-UR, Droevendaalsesteeg 1, 6708 PB, Wageningen, the Netherlands (e-mail: marcel.toonen@wur.nl) [2]Genetwister Technologies, Nieuwe Kanaal 7b, 6709 PA Wageningen, the Netherlands (e-mail: m.j.m.ebskamp@genetwister.nl) [3]Institute of Applied Research at Reutlingen University, Alteburgstrasse 150, 72762 Reutlingen, Germany (e-mail: robert.kohler@reutlingen-university.de)

1. Introduction

Plant fibres have decisive advantages compared with synthetic fibres. One great advantage of plant fibres is their optimized strength to weight ratio. Others are their better workability as a result of optimum fibre length and cell wall thickness, their high anisotropic qualities and their good ion exchange capacity. The natural products are readily biodegradable and renewable.

Innovations in fibre applications are underway e.g. in biobased composites, specialised (hybrid) non-wovens and others. In these areas bast fibres play an important role. For the various applications, it is important to gain control over fibre characteristics, which in turn are determined by cell wall composition and interaction of cell wall components. Functional genomics projects are running to develop a comprehensive understanding of the genetic mechanisms that control *in planta* cell wall development and, consequently, regulate important fibre properties.

P. Ranalli (ed.), Improvement of Crop Plants for Industrial End Uses, 155–180.

This chapter will focus on the latest developments in bast fibre research and its applications in composite materials.

2. State of the art

2.1 Plant fibre development

Plant fibres originate from various tissues in a plant. Cotton fibres are elongated cells of the seed epidermis while wood and bast fibres originate from the vascular bundles in the plant stem. Based on the plant origin, fibre development follows distinct pathways that result in specific technical properties for each type of fibre.

Wood fibres are tube-like lignified and dead cells. A typical softwood fibre is 2-4 mm long and 20-40 μm wide. A typical hardwood fibre is 1.1-1.2 mm long and 14-40 μm wide. The formation of wood fibres is an ordered developmental process involving cell division, cell expansion, secondary wall deposition, lignification and programmed cell death (reviewed by Mellerowicz et al. 2001). Vascular cambium cells give rise to secondary xylem or phloem, vessel elements or fibres. In the xylem, fibre and axial parenchyma cells expand primarily in the radial directions whereas vessel elements may undergo substantial tangential growth. Fibre elongation is achieved by intrusive tip growth. Because various cell types follow different developmental pathways, mechanisms that regulate the whole development must be in place.

After radial expansion is completed secondary cell walls are formed by the formation of dense array of helical, almost transverse cellulose microfibrils (S1 layer). Successive layers of the secondary cell wall have orderly arranged cellulose microfibrils. Vessel cells are joint end-to-end to form a functional vessel to ensure root to shoot water transport. Cell wall formation in the pit-perforation area is quite distinct from the other cell walls. Lignification starts in the middle lamella, particularly in cell corners where cells have completed the deposition of the S1 layer. Lignification progresses inwards concomitantly with cellulose, mannan and xylan deposition. When lignification is complete vessel cells undergo programmed cell death that involves the hydrolysis of the protoplast.

Bast fibres are cellulose rich cells derived from vascular bundles that display high mechanical properties. Bast fibres used for industrial purposes originate from the procambium and correspond to sclerenchyma phloem cells. In the plant stem, individual cells (elementary fibres) are intimately associated through their middle lamellae to form fibre bundles, situated

parallel to the longitudinal axis of the stem. The elementary fibres are 20 to 100 mm in length and 15-25 μm in diameter. Generally, secondary fibres are shorter and thinner.

Bast fibres are characterized by thick secondary cell walls that at maturity almost completely fill the lumen. The cell walls are rich in cellulose and contain moderate levels of hemi-cellulose and contain lignin levels between 3 and 8% in hemp (Crônier et al. 2005; Toonen et al. 2004) and 1.5 and 4.2% in flax (Day et al. 2005). Although pectin is only present in low amounts in the middle lamella it is considered important as a 'cementing material' to form the fibre bundles (Girault et al. 2000). In flax it has been suggested that the cementing material is reinforced by the presence of various 'phenolic compounds' (Sharma et al. 1999).

Differences in fibre quality can be attributed to differences in crops and used varieties, agronomic conditions and environmental influences like weather conditions. In general, fibre diameter decreased with increasing plant density. Compression stress e.g. caused by wind load or growth stress may lead to fibre dislocations, already during the growth of the plant (Thygesen and Hoffmeyer 2005).

2.2 Plant cell walls

The characteristics of plant fibres are determined by the chemical and morphological composition of the cell wall. This wall is composed of three independent but interlacing networks of cellulose-hemicellulose, pectin and structural glycoproteins, respectively. The cellulose-hemicellulose network is the principal load-bearing element in the primary cell wall. The secondary cell wall contains cellulose and non-cellulosic cross linking glycans e.g. xylan (Awano et al. 2002) and lignin, together with a variety of proteins and other minor components. The lignin presumably binds cell wall polysaccharides through both covalent and non-covalent interactions to form a lignin-polysaccharide complex. According to fibre type, these non-cellulosic substances can add up to 30% weight in bast fibres and more than 50% in wood.

Cells of dicotyledonous plants are composed of type 1 cell walls, which are characterized by a cellulose-xyloglucan framework with approximately equal amounts of cellulose microfibrils and xyloglucans. The cellulose-xyloglucan framework is typically embedded in a network of abundant pectic polysaccharides, which comprise homogalacacturonases, rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II). Current models suggest that these three components are covalently linked to each other, thereby forming a pectic network (Willats, et al. 2001; Ridley et al. 2001).

Type II cell walls are predominantly found in monocotyledons, which include cereals and miscanthus. Compared to type I cell walls, type II cell walls contain less xyloglucans and pectin. The predominant glycans that cross-link the cellulose microfibrils are glucoronoabinoxylan and mixed glucans.

2.2.1 Molecular control of biosynthesis and texture of cell walls

The molecular study of plant cell wall biosynthesis is still limited. Recent studies, in particular those exploiting Arabidopsis molecular genetics, have identified small sets of genes involved in the synthesis of cellulose synthesis (Roberts and Roberts 2004), pectin (Bouton et al. 2002), xyloglucans (Faik et al. 2002; Madson et al. 2003; Vanzin et al. 2002) and galactomannans (Liepman et al. 2005). Polymerization of cellulose is carried out by the membrane bound rosette complex, which most likely comprises 3 different cellulose synthase catalytic subunits. Two sets of three evolutionary conserved cellulose synthases are required for respectively primary cell wall deposition in growing cells and secondary cell wall thickenings in xylem cells. In addition to the cellulose synthase genes, a membrane-bound endo-1,4-glucanase, a secreted chitinase-like protein, an unknown membrane protein, and a GPI-anchored protein are required for cellulose synthesis.

2.2.2 Mechanical properties of cell walls related to industrial applications

The structure of the primary cell wall components has been elucidated to some extent, however, little is known about the mechanical function of the individual polymers. Tensile property measurements of dark grown wild-type and mutant Arabidopsis hypocotyls (Pena et al. 2004; Ryden et al. 2003) have shown that several cell wall components, for example, galactose-containing side chains of xyloglucan, and the cross linking of pectin by means of borate-complexed rhamnogalacturonan II, contribute to the overall stiffness and strength of the organ and thus the mechanical performance of the cell wall. In terms of secondary cell walls, several investigations showed the importance of cellulose orientation in the cell wall (Burgert et al. 2004) and the mechanical contribution of the matrix polymers (hemicelluloses and lignin).

Mechanical characterization of plant cell walls at the micro- and nanoscale demands sophisticated tools for sample preparation and testing. Using plant tissues to investigate the mechanical properties of cell walls does not allow distinctions to be made between deformation mechanisms inside the cell-wall from those mediated by cell-cell interactions. A chemical

treatment is non-specific and will result in fibres that are not only separated at the middle lamella, but also cell wall polymers will be partly degraded. Therefore, chemical treatments cause major difficulties when investigating the mechanical relevance of the systematic modification of the ratio of cellulose fibrils and matrix polymers.

2.2.3 Modelling of the cell wall, the geometrical model for cellulose microfibril orientation

The cell wall texture is a composite of cellulose microfibrils arranged in one cellulose microfibril thick lamellae. The orientation of the cellulose microfibrils within a lamella is constant, but may vary between lamellae. The most striking texture is the helicoidal wall, which consists of subsequent lamellae in which the orientation of the cellulose microfibrils changes by a constant angle. Other wall textures are the axial, helical, crossed-polylamellate, transverse and the random wall textures, and combinations of these. Since wall texture is cell type and developmental stage specific, its development must be highly regulated by the cell it embraces. It has been proposed (Emons and Kieft 1994) that the default mechanism, which determines the orientation of cellulose microfibrils as they are deposited in the absence of other influences, is geometrical in origin. Based on the observation that cellulose microfibrils always appear approximately evenly spaced in close-packed lamellae and that their average distance apart does not depend on their orientation with respect to the cell axis, the geometrical close packing rule was formulated (Emons and Kieft 1994):

$$\sin \alpha = \frac{Nd}{2\pi R}$$

This formula relates the cellulose microfibril winding angle to the number of cellulose microfibrils being deposited, the distance between them and the radius of the cell. This explicit mathematical rule is the corner stone of a dynamic developmental model, which rests on the assumption that new active cellulose synthases insert into the plasma membrane through exocytosis of Golgi vesicles, or else, are activated within moving localized regions along the cell, the cellulose synthase activation domains. Once activated in the plasma membrane, the cellulose synthases move forward propelled by the forces generated in the cellulose microfibril deposition / crystallization process. In the course of time, their angle of motion with respect to the cell axis is continuously adapted to the changing number of other cellulose synthases in their neighbourhood in order to satisfy the geometrical close packing constraint. The cellulose microfibrils deposited follow the tracks of the cellulose synthases and as such constitute a

'recording' of their motion. The final ingredient of the model is that cellulose synthases have a finite active lifetime.

The geometrical model provides a conceptual framework for the alignment mechanism of cellulose microfibrils, which unites examples where cortical microtubules are and are not parallel to nascent cellulose microfibrils, and in which they do not directly move or channel the synthases but may be involved in their activation inside the plasma membrane. The model also enables function based design in model species using transgenic plants. This approach will lead to better understanding of the relationship between cell wall texture, chemical composition and mechanical properties and gene expression, thereby enabling breeding of crops for novel industrial applications.

2.3. Retting and decortication

After harvesting of the stems of fibre plants like flax and hemp the bound between individual fibres is loosened by a process called retting. subsequently processing is required to separate the bast fibres from the woody core of the stem. To satisfy the demand for high quality plant fibres, several methods have been developed to process the plant stems. High quality fibres can be obtained from water retting or enzymatic retting though in Western Europe only dew retting can be economically applied. During the process of dew retting cell wall components are degraded by enzymes produced by micro-organisms. Due to the fact that the process can hardly be controlled and dependent on external conditions like weather the resulting fibre quality is very variable. To overcome this, new machinery has been developed for the decortication (scutching) of green, non-retted flax. The resulting fibres were very coarse and contained high amounts of adherent residual shives. In addition, the high mechanical impact resulted in kinks and flaws in the cellulosic structure leading to highly damaged fibres. In various European countries experiments have been carried out with chemical retting on the field e.g. with sulphur dioxide or retting on the stem by spraying with round-up. None of these efforts came to a final application due to increased coefficients of variation due to inhomogeneous retting, lack of specificity and high costs. Also the trials using enzymes for retting were not successful because they were not specific for the desired actions: removal of the cortex and release of the fibre bundles.

2.4 Fibre requirements for composites

2.4.1 Inherent fibre properties

After decortication cellulosic fibres are obtained. Their most important properties with respect to composites are mechanical strength, thermal stability and the interaction with water. The fibre structure and composition is specific for the plant and depends on the growth conditions, maturity and location within the plant. For reinforcement, the tensile strength of the fibres should be as high as possible and the modulus of elasticity (MOE) of the fibres should exceed the modulus of the matrix at least by a factor of three. The natural fibres are by themselves complex composites, and eventually removal of non-cellulosic compounds has to be considered with respect to any application.

Fibre tensile strength is directly correlated to cell wall properties. A reduction in cellulose content in Arabidopsis hypocotyls leads to a decrease in tensile strength and modulus. Based on studies on the Arabidopsis mutants MUR2 and MUR3 which contain mutations in genes encoding xyloglucan-specific fucosyl and galactosyl transferases it was concluded that the xyloglucan-cellulose network as well as RGII borate complexes determine the tensile properties of the fibre (Ryden et al. 2003).

The mechanical strength of the elementary fibres also depends on the number of inherent imperfections, which are greatly influenced by the different steps of the fibre extraction process and the further treatments. As described before, the anisotropic structure of the fibres is sensitive to transverse forces. Compression or bending can induce internal dislocations (also referred to as ‘kinks’) where the fibres will preferably break under load (Kohler and Kessler 1999).

All these factors give rise to a large variation in properties. In fact the measured fibre strength is the superposition of the cohesion of the fibrils, their adhesion to the natural matrix and the kind and number of defects. The general result is that the tensile strength of fibres increases with fibre fineness, and decreases with increasing distance between the test grips. The relations can be described by Weibull statistics accounting for the probability for the presence of defects (Dill-Langer et al. 2003; Gutowski 1997; Lee 1993). As a consequence the mechanical testing of natural fibres has to be done at different distances between the grips to account for the imperfections. Published values are only comparable when the clamping length and the crosshead speed are reported. Although the most exact results are obtained by single fibre measurements, the big variations make it difficult to gain representative figures by this laborious method. Measuring

fibre bundles or other arrangements are more practical to give averaged results, but suffer from the confounding of additional factors (e.g. the bundle effect: in bundles the fibres break subsequently so that the initial cross-sectional area decreases and the measured stress at break is too low (Harig et al. 1994).

On the average, the specific tensile strength of the strong bast fibres is comparable to glass fibres, but the best values found by single fibre measurements are much higher and almost comparable to aramide fibres. At present there is no process available to separate just the strongest fibres from the bulk, and even if it were, the yield would be much too low to be economic. So other strategies are needed for achieving improved composite properties.

The thermal stability of the fibres is another important feature with respect to compounding and the intended applications as well. Thermal degradation of cellulosic fibres depends on their composition. Pure cellulose has the highest stability. Decomposition starts at about 150°C beginning with elimination of water and discoloration. Beyond 220°C mechanical strength drops rapidly with time. Under nitrogen the peak degradation temperature is about 350°C (Kohler and Kessler 1999). Oven storage experiments in air have shown that temperatures of 200°C for 10 minutes or even up to 220°C for about 3 minutes produce only a minor reduction of the tensile strength (Kohler et al. 1997).

Cellulosic fibres are hydrophilic. The water uptake from the vapor or liquid phase is a crucial factor with respect to the formation and the properties of composites. Interaction with water, generally characterized by sorption isotherms, depends on the content of hemicellulose, pectin and lignin and can be controlled by specific modification (Kohler et al. 2003; Sreekala and Thomas 2003).

2.4.2 Fibre reinforcement

In order to obtain high quality composites the following points should be addressed:

Fibre shape

In composites with fibres of finite lengths, applied tensile stress is transferred from the matrix to the fibre as shear stress. The stress transfer starts at the fibre ends and increases symmetrically along the interface. The critical fibre length L_c is the minimum fibre length necessary for transferring sufficient stress to reach the tensile strength of the fibre so that it breaks. L_c is minimal in the case of perfect adhesion. It can be determined experimentally e.g. by the single fibre fragmentation test (Kim and Mai 1998), and taken as a measure for the actual fibre-matrix adhesion.

With increasing tension and sufficient adhesion the fibres are more and more fragmented to their critical length or shorter. The elongation of the sample is given by the elongation of the matrix in the space between the fibre ends, and with increasing fragmentation the MOE decreases due to this "end effect" (Stokes and Evans 1997). Adhesion is never perfect and with further elongation of the sample the fibres will delaminate from the matrix and finally the material will fail.

For "short" fibres with limited length indeed the aspect ratio (length divided by thickness) is the effective parameter, i.e. the finer the fibres the shorter they can be to achieve the same reinforcement, a very important consequence with regard to nanosized fibrils or whiskers. As a general rule the aspect ratio for maximum strength and modulus should be about 100. For fibres with quasi infinite length the elongation of fibres and matrix are equal and due to the small elongation of the fibres the MOE is high. As long as the fibre length exceeds 5 L_c the "end effect" is negligible and the modulus approaches that of continuous fibre composites (Stokes and Evans 1997).

Fibre array and orientation

The crucial factor is the angle between the direction of applied stress and fibre orientation. When both are parallel (0°) maximum strength and modulus are obtained. The strength of composites with unidirectionally oriented fibres stressed perpendicular to the fibre axis is lower than the strength of the matrix alone (Gutowski 1997).

Loose fibres are in most cases randomly distributed providing isotropic properties. Rovings or yarns allow for oriented, especially unidirectional arrangement. The fibres are assembled parallel and held together by surface contact or finishes. In non-woven mats the fibres can be randomly distributed or oriented to a certain degree and bonded by different mechanisms. Woven or interlaid fabrics provide defined orientation. An important feature of mats and fabrics is the drapeability, necessary for the molding of three-dimensional forms (Gutowski 1997; Lee 1993).

Fibre volume fraction

The reinforcing effect of the fibres increases with increasing fibre content. Depending on fibre arrangement there is a maximum volume fraction that allows for a complete embedding into the matrix. With higher fibre content the matrix cannot fill all voids and the strength is reduced by the resulting pores (Stokes and Evans 1997).

2.4.3. Fibre matrix adhesion

The adhesion between fibres and matrix is decisive for the stress transfer, but also for energy dissipation on impact (Kim and Mai 1998; Stokes and Evans 1997).

The adhesion is widely governed by secondary chemical bonds, i.e. van der Waals forces and acid-base interactions. Both are directly related to the specific surface of the fibres and the surface energy of fibres and matrix respectively. Excellent wetting of the fibres is needed to achieve closest contact necessary for establishing strong bonds.

Primary, covalent bonds are desirable because of their high bond energy. They need special functionality of fibres and matrix or the addition of functional coupling agents.

Actually the interphase has a finite thickness and should rather be addressed as "interphase", but little is known about its structure and properties. The character of the interphase and the understanding of the mechanisms of adhesion and energy dissipation are eminent topics of the present research (Bismarck et al. 2002; George et al. 2002; Gutowski 1999; Pott 2004).

2.5 Composites

2.5.1 Matrix materials

Matrix materials are predominantly polymers from all three classes, thermoplastics, thermosets and elastomers, but also inorganic materials are important.

Thermoplastic polymers are ductile, easy to process by thermoforming, injection moulding and extrusion and simple to recycle. On the other hand thermoplastics have a high melt viscosity, which makes fibre wetting difficult and gives rise to increased shearing forces. Processing temperature and time and eventual shearing must be controlled so to not degrade the fibres. The mechanical strength of thermoplastics is limited and drops significantly above the glass transition temperature T_g.

At present polypropylene is the preferred thermoplastic matrix material. Polypropylene combines good mechanical properties with acceptable low processing temperature, low cost and ease of recycling, but poses problems with respect to fibre wetting and adhesion between the apolar matrix and the polar fibres. Present efforts aim at improved compatibility by the use of coupling agents or the modification of the components. PVC and mainly

polyethylene are used for wood fibre composites. Other thermoplastics are investigated but have not yet found broader industrial application.

Thermosets provide good wetting of the fibres due to the low viscosity of the monomers. The fibre-matrix-adhesion is usually good and the mechanical and thermal properties are excellent due to the cross linked structure. On the other hand the processing is more demanding and recycling is restricted. Thermoset matrices are necessary whenever increased mechanical strength, chemical and thermal stability are needed. The most used thermosets today are unsaturated polyesters, epoxy resins, polyurethanes and acrylics. Phenolic resins have been widely used because they combine good compatibility with cellulose and low flammability, but are outdated in car applications.

Elastomeric matrices require excellent interfacial bonding because of the extreme differences in elongation. Natural fibre reinforced elastomers might be promising with respect to the upcoming nanosized fibres.

Biopolymers as renewable and biodegradable materials are of increasing interest. Beside industrial scale products such as starch, natural latex or the well-known cellulose esters, there are various biological raw materials under investigation which will gain importance for novel products as described below.

2.5.2 Composite processing

Composites are prepared by the existing approved methods (Gutowski 1997), but the special properties of the natural fibres need adapted processing. There are two main routes of manufacturing thermoplastic composites:

Extrusion
Compounding the fibres with thermoplastic polymers in an extruder. The molten compound is cooled and granulated, whereas the fibres are shortened at least to the length of the granules.

Long-fibre thermoplastics (LFT) (Anonymous 2006; Henning et al. 2005; Van Hattum et al. 2005), for high performance composites are produced by two variants. (1) The unidirectional oriented fibres are impregnated forming a rope which is cut into long granules of 11-25 mm. (2) In new specialized D-LFT production lines thermoplastics and fibres are compounded in a twin-screw extruder and the melt is directly delivered to a hot press or to a modified injection moulding machine (Anonymous 2006; Henning et al. 2005). The challenge for using natural fibres in these processes is to assure continuous and homogeneous dosage in a form that is easily opened and distributed in the polymer melt. The fibres are either fed to the extruder in the form of tow or roving, or special devices must be

developed which can handle loose fibres, open entangled structures and allow for a continuous dosage from stock.

Non-woven sheets
Semi-finished non-woven sheets or mats with intimately blended natural and thermoplastic fibres (e.g. polypropylene) can be directly used for the production of parts by compression moulding with established procedures. Thermoset composites are mainly prepared by producing non-woven mats from the natural fibres, followed by impregnation with thermoset resins. In-line compounding with thermoset resins, analogous to D-LFT can be expected.

3. Towards novel applications

3.1 Challenges for in planta fibre modification

3.1.1 Cell wall genomics

The cell wall as the main building block of plant fibres is a complex structure. During plant development it is a metabolically very active compartment. Interlacing molecules are cleaved and reconnected and novel molecules are deposited in the cell wall. The complexity of the biosynthesis and modification of the cell wall molecules is probably best illustrated by the number of genes involved. Carpita et al. (Carpita et al. 2001) estimated that 15 percent of the *Arabidopsis* genes are directly involved in cell wall metabolism. In studies on genes expressed in poplar and flax, around 4% of the genes were attributed to cell wall metabolism (Boudet 1998; Day et al. 2005; Sterky et al. 2004). Yokoyama and Nishitani (Yokoyama and Nishitani 2004) classified the cell wall genes into 32 gene families. These families can be attributed to molecular processes like the biosynthesis and modification of cellulose, callose, glucans, pectin, lignin and structural proteins and the cross linking between the various cell wall molecules. If we learn how these processes are controlled we can modify fibre properties in planta. During the last decade, plant genomics studies have been initiated to understand the genetic regulation of cell wall metabolism.

Due to its economic importance as a forestry species and its characteristics as a model species, Poplar (*Populus* spp.) has extensively been used for genomic studies. *Populus* species have a small genome (five-fold the size of *Arabidopsis*), grow fast, can easily be propagated and can be

transformed. A significant contribution to the poplar community was the recent sequencing of the poplar genome, and annotation programs estimate the number of genes to be about 40,000. An expressed sequence tag (EST) database with 101,019 sequences from 19 different libraries (Sterky et al. 2004) and a microarray with 16,839 unigenes (http://www.populus.db-umu.se) have been produced. Twenty three percent of the ESTs have no homologues in the various databases and a subset of these may encode novel functionalities in cell wall biosynthesis. Mainly by sequence homology with Arabidopsis genes, over fifty percent of the ESTs could be attributed to a known function. This has enabled the identification of major carbohydrate active enzymes in wood forming tissues, including enzymes involved in cellulose and hemicellulose biosynthesis and remodelling (Aspeborg et al. 2005). Wood-specific enzymes from large gene families such as expansins and xyloglucan endotransglycosylases (XET) have also been characterized. In addition to characterization of genes, genetic maps have been made for several Populus species and quantitative trait loci (QTLs) have been mapped which will allow breeding for specific fibre traits.

For flax a set of 927 sequences has been published of which 4.4% could be attributed to cell wall biosynthesis (Stokes and Evans 1997). For hemp the expression of genes during plant development has been studied. Genes involved in lignin metabolism could explain major differences between the woody core and the bast fibre (Van den Broeck et al.). The expression of over 3000 genes in hemp fibre tissue was correlated to the chemical composition of the tissue by an integrated statistical analysis. Expression of 58 different genes showed a high correlation to various chemical characteristics of the cell wall (Reijmers et al. 2005). Further research is required to elucidate the function of the various genes in specific biosynthetic pathways.

Although the identification of new genes involved in cell wall biosynthesis emerges rapidly, it is surprising to see how little (molecular) information is available about cell wall biosynthesis. Extensive research is required to elucidate the role of biosynthetic enzymes in every aspect of cell wall biosynthesis. It can be foreseen that combining the data obtained from genomics and proteomics studies in the various model systems (Arabidopsis, Zinnia, Cotton and Populus) will give novel insights in the biological processes that underlie cell wall formation. These results can be used for applied fibre research. A number of examples are elucidated in the next section.

3.1.2. Biotechnological approaches

Important aspects for composite applications are the length and diameter of the fibre and physical properties of cellulose, including the degree of polymerisation, crystallite size and microfibril angle. These aspects also apply to textile fibres.

Cellulose based biocomposites are limited by poor interfacial binding between the polyhydroxyl fibre surface and the hydrophobic matrix component. Gustavsson showed that *in vivo* modification of fibre surfaces could alter surface properties and therby binding to matrix materials (Gustavsson et al. 2004; Gustavsson et al. 2005; see also section 3.3.2). Applied *in planta*, such an approach would yield a whole new range of functional groups on natural fibres.

Cell length might be modified by expansins. These proteins have unique cell wall loosening properties and have demonstrated effects on the mechanical properties of plant cell walls. They appear to play a central role in the control of plant growth. Expansins are thought to mediate cell wall extension by disrupting hydrogen bonds between cell wall glycans and cellulose microfibrils, thus allowing the microfibrils to slip. *In vitro* treatment of plant cell walls with expansins results in their extension (McQueen-Mason and Cosgrove 1994). Experiments using transgenic plants have indicated that it is possible to modulate cell expansion through manipulation of expansin expression (Zenoni et al. 2004). It may be hypothesized that high expression of expansins in fibre cells will increase cell elongation and thus yields longer and thinner fibres that are of high interest to the fibre industry.

New functionalities can be created by the production of biodegradable polymers inside a natural fibre. Introduction of low amounts of polyhydroxybutyrate (PHB) in the lumen of flax fibres already resulted in significant modifications in Young's modulus *E*. (Wróbel et al. 2004). With improved gene constructs and additional research this approach shows high potential for various applications e.g. in the field of composites where the inherent polymer promises improved adhesion and strength.

3.2 Fibre modification for improved products

On a technical scale fibre bundles can be isolated (e.g. by decortication) and subsequently refined by approved processing methods (e.g. steam explosion (Kessler 1998) Duralin®-process (Pott 2004), scouring/-mercerization (Gassan and Bledzki 1999) or the use of additives). The current research concentrates on specially designed combinations of chemical, thermal and mechanical fibre treatment for very substantial modifications

of fibre surface and structure (Azizi Samir et al. 2005; Gindl and Keckes 2005; Kvien et al. 2005; Mathew and Dufresne 2002; Pöhler et al. 2004; Prasad et al. 2003). Processes with dissolved cellulose in homogeneous phase will not be considered here.

The objectives of fibre surface modifications are increased compatibility and adhesion to the matrix and reduced hydrophilicity. Because fibre modification is done by heterogeneous reactions, the effects are limited to the fibre surface and a certain zone accessible to the molecules, whereas the core of the fibres remains basically unchanged. Therefore the fibres always preserve to a certain extent the genuine properties of cellulose, mainly its hydrophilicity. These effects can be limited by technical modification or modification of fibres *in planta.*

3.2.1 Decortication / scouring / mercerisation

Isolation of elementary fibres is hampered by the strong adhesion between the fibres in the plant stem. This adhesion might be loosened *in planta* by modification of the cell wall biosynthesis or during the retting process by the application of specific enzymes. However, due to the specific composition of the cell walls in the different tissues specific enzymatic treatments will be necessary for every step in the isolation of the fibre bundles and elementary fibres. In the cortex the main adhesive molecules are the calcium pectates. These can be hydrolysed using polygalacturonase or pectate lyase resulting in dissociation of the cortical tissues. The interface between the fibre bundles (phloem cells) and core (cambium cells) is characterized by a large variety of polymers, including some pectins but also hemicelluloses such as xylans and xyloglucans. To date it is not clear which polymers contribute most to the adhesion between both tissues. The xylans might be one of them but it is likely that also other components (especially phenolics) are involved in the cross-linking. The adhesive components in the elementary fibres are also pectins but with a different structure in comparison to the cortex. Specific pectinases including galactanases have been shown to be able to degrade these carbohydrates thereby releasing the smaller fibre bundles and finally the elementary fibres. However, phenolic compounds still hamper the total release of the elementary fibres.

Due to the fact that these cell wall components only contribute to 1% of the total cell wall materials the identification of these compounds will be challenging.

Although scouring and/or mercerization are already present for a long time, it are still important methods for improving reinforcement fibres. The alkaline treatment removes most of the hemicellulose and pectin, helps to

liberate single fibres and increases their specific surface by roughening (Bismarck et al. 2002; Gassan and Bledzki 1999; Kozlowski and Wladyka-Przybylak 2004). Furthermore, depending on tension, the fibre structure is changed, accessibility is altered and mechanical strength is improved.

3.2.2 Enzyme treatment

Results achieved by fibre treatment with various enzymes reach from fibre bundle disintegration and scouring effects (e.g. by pectinases, xylanases), surface smoothing (cellulases) to chemical surface modification and improved adhesion (laccase in lignocellulosic systems (Felby and Nielsen 1997; Ossola and Galante 2004; Widsten 2002). Although enzymes are increasingly available in industrial amounts at reasonable cost, their use for improvement of natural reinforcing fibres is still subject to research and development. The potential of the work is shown by the modification of cellulose fibres by lipase B. Derivatisation to make the cellulosic fibre more hydrophobic leads to degradation of the cellulose structure and disruption of the hydrogen bond network. Enzymatic modification of the cellulosic fibres is more gentle and reactions can be carried out more specifically. A specific lipase B is able to modify fibre surfaces. The protein fused to a cellulose binding domain (CBD) catalysed ring-opening polymerisation on fibre surfaces (Widsten et al. 2004). Hydrophobicity of the fibre surface arose from lipase-catalysed polymerisation of externally applied ε-caprolactone. The polymer was attached to surface deposited polymers. A similar approach was used to modify xyloglucan. Regioselective acetylation of xyloglucan oligosaccharides (XGOs) was catalysed by the *C. antarctica* lipase B. The modified XGOs could be incorporated into xyloglucan chains by a xyloglucan endotransglycosylase (XET). The resulting xyloglucans exhibited a high affinity for cellulose surfaces which resulted in fibres with altered surface properties (Gustavsson et al. 2005).

3.2.3 Cationization

In water cellulose has a negative surface charge. By reaction with agents containing quaternary amino groups the surface is rendered cationic. Cationized cellulose acts as ion-exchanger and has been demonstrated to successfully remove complexed heavy metal ions such as chromate from waste water (Hashem et al. 2003).

3.2.4 Coupling agents

The simplest way for improving the adhesion between the polar, hydrophilic cellulose fibres and the apolar polymer matrix is by addition of compatibilisers, adhesion promoters or coupling agents. These substances function by reducing interfacial energy and enabling additional secondary or primary bonds between themselves and fibres and matrix respectively. Secondary bonds are established for instance by amphiphilic molecules which undergo hydrogen bonding to the OH-groups of the cellulose whereas their apolar part interacts with the matrix by dispersion forces. Long chain polymeric amphiphiles are more effective than short molecules due to an increased interface.

Strong covalent bonds to both components are obtained with reactive coupling agents (Bismarck et al. 2002; George et al. 2001; Kozlowski and Wladyka-Przybylak 2004; Gassan and Bledzki 2000). For this the matrix must have functional groups. Typical examples are:

- MAPP (Polypropylene modified with maleic anhydride) where the anhydride can form covalent ester bonds with OH-groups
- reactive silanes, which after being hydrolysed to silanols bond to OH-groups, and the reactive part (e.g. vinyl or amino group) reacts with unsatured polyester resin or epoxy resin respectively
- polymers with isocyanate groups which react with active hydrogen atoms

New considerations indicate that long chain molecular entanglement is more important than just covalent bonds (Gutowski 1999). Coupling agents can be applied to the fibre surface first, added to or copolymerized with the matrix polymer prior to or during the preparation of the composite. For best effects the proper choice of application method and reaction conditions is mandatory.

3.2.5 Chemical surface modification / surface grafting

Analogous to the reaction of coupling agents with the fibre surface, the fibres can be subject to different chemical reactions that alter their surface polarity and surface energy. A well tested example is the acetylation of cellulose (George et al. 2001; Kozlowski and Wladyka-Przybylak 2004; Zafeiropoulos et al. 2002) making the surface apolar and hydrophobic. Different from the production of cellulose ester polymer in solution, surface grafting is a heterogeneous reaction at the interface where only the accessible OH-groups will react under special conditions. Also other reactions with the OH-groups can be used to covalently bond molecules to the fibre, thus offering a broad range of possible surface modification.

3.2.6 Corona / Plasma / UV treatment

The dry "physical" treatment of fibres in a plasma of ionized gas, generated by corona discharge at atmospheric pressure or in a cold plasma under reduced pressure, is very appealing because drying processes and waste water problems are avoided (Gassan and Gutowski 2000; Yuan et al. 2004). Whereas corona discharge induces only reactions with ionized air, mainly oxidation, the treatment in a low pressure plasma chamber is more effective and offers a broad range of different reactions with different gases including surface graft polymerisation when using monomers. Furthermore in cold plasma there is always high energetic UV radiation present that contributes to the surface reactions. UV radiation in air generates radicals and results in oxidation, but in combination with wet chemistry high energy radiation allows for grafting reactions e.g. with acrylic monomers. Corona treatment and UV radiation of jute increased the polar component of surface free energy and improved the adhesion to an epoxy matrix, but led to a decrease of fibre strength (Gassan and Gutowski 2000).

3.2.7 Fibrillation

The use of nano-sized fibres is presently the most innovative and promising approach for the generation of high performance composites, and efforts are increasingly undertaken to develop suitable processes (Azizi Samir et al. 2005; Gindl and Keckes 2005; Kvien et al. 2005; Mathew and Dufresne 2002; Pöhler et al. 2005; Prasad et al. 2003). Fibrillation means disintegration of the whole fibres and liberation of the microfibrils from the different S-layers. The idea is to get rid of the confounded effects of cellulose strength, adhesion between microfibrils and existing defects, and directly use the highly crystalline microfibrils with their high aspect ratio for the formation of strong nano-composites.

4. Novel products

To develop novel composite materials and to develop novel markets for biobased products, intelligent combinations of the factors described, choice of fibres and matrix, fibre preparation and processing technology are required. This section highlights some of the latest developments.

4.1. Biobased matrix materials

An important field for the development of novel products is the use of biological matrix polymers. Especially thermoplastic biopolymers from renewable resources, such as polylactide (PLA) (Wallenberger and Weston NE 2004; Oksman and Selin 2003) and polyhydroxyalkanoates (PHA) are most promising. The biopolymers can be blended with synthetic biodegradable polymers to achieve different properties and still preserve biodegradability.

Especially PHA is in the focus of research (Peterson et al. 2002). These thermoplastic polymers are generated by bacteria and are obtained in industrial amounts from biotechnological processes. By genetic engineering production of PHA has been successfully transferred to plants (Poirier et al. 1992). However, significant additional efforts are needed to create plants of commercial interest. These include detailed studies on the changes in metabolism created by the expression of the introduced genes (Snell and Peoples 2002). As described before, expression of PHA in fibres may open totally new possibilities for natural fibres and composites.

Reactive substances from biological raw materials are also increasingly used for thermoset matrices (e.g. curing triglycerides (O'Donnell et al. 2004) or as components such as natural polyols in polyurethane formulations (Lichtenthaler and Peters 2004; Marsh 2003). An important feature of some biopolymers is biocompatibility, which make them useful for biomedical applications.

4.2 Nanocomposites

Presently many efforts aim at the development of high performance composites with greatly improved strength by the use of nano-sized cellulose fibrils ("whiskers"). These are microfibrils liberated from plant fibres or cell-walls or more exotic fibrils such as tunicin whiskers from sea animals (Azizi Samir et al. 2005; Gindl and Keckes 2005; Kvien et al. 2005; Mathew and Dufresne 2002; Pöhler et al. 2004; Prasad et al. 2003). Due to the high crystallinity, high aspect ratio and almost complete absence of defects, the tensile strength of such fibrils is near the theoretical strength of cellulose and almost comparable to aramide. Significant improvement of composite strength is obtained even with fibre loads below 10%.

"True" nanocomposites are still in the research stage, mainly because of two problems: (1) Up to now the extraction of intact fibrils from the fibre or cell walls has only be achieved by very time consuming multi-stage processes. (2) Presently the fibrils can only be held separate in diluted water suspension. In more concentrated suspension and on drying the fibrils

entangle and adhere strongly together so that there is still no pragmatic way to re-separate and homogenously distribute them in hydrophobic polymer compounds.

More related to practice is a partly fibrillation of the S1 layer of bast fibres by subsequent processes comparable to common textile scouring, which can be realized on existing finishing machinery (Kohler and Nebel 2005; Nebel and Kohler 2005). As a result the specific surface is extremely increased although most of the fibre cores are preserved. Again the entanglement of the fibrils poses a problem for polymer compounding, but composites with considerably improved mechanical properties have been successfully prepared from impregnated wet-laid sheets.

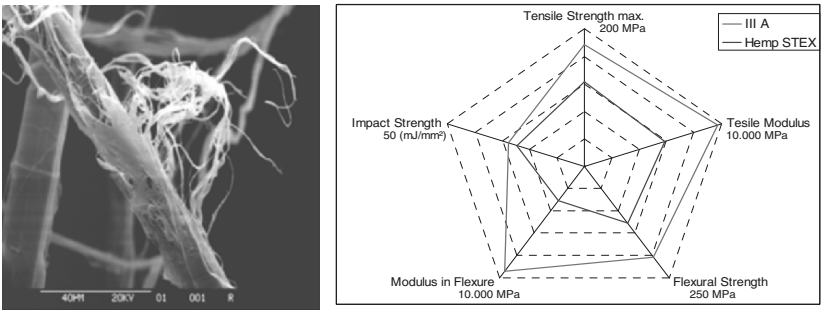


Fig. 6.1. a) SEM micrograph of a partly fibrillated S1 layer of a hemp fibre. b) Comparison of mechanical properties of epoxy resin composites reinforced with 70% cellulose fibres steam exploded hemp (Hemp STEX) vs. fibrillated hemp (III A).

4.3 Micro- and nano-fibrillar structures

In addition to the nano-composites the cellulose fibrils offer great prospects for innovative products. Possible applications are the replacement of micro-glass fibres in filter materials, which can then be composted or in cinerated, biodegradable adsorbents or ion exchangers, non-woven sheets for special textiles, scaffolds for tissue engineering, precursors for biomorphic ceramics etc.

4.4 Biobased carbon-fibre structures and ceramics

By pyrolysis of cellulose or preferably lignocellosic materials carbonization occurs whereby the process conditions determine the preservation of the original morphology (e.g. wood, non-woven or woven fabrics, corrugated cardboard, etc.) which can then be used as template for biomimetic structures (Popovska et al. 2005). The carbonized materials can possibly be used for thermal resistant products such as insulation material, filters, friction materials, electroconductive products etc.

Porous activated carbon with extremely high specific surface can be produced and used as filter material or adsorbents.

Last but not least the carbonized cellulose structures can be converted into biomorphic ceramics by melt or vapor infiltration with inorganic substances forming carbide ceramics. Examples are chemical vapor infiltration with $SiCl_4$ / H_2 for SiC and $TiCl_4$ / H_2 for TiC ceramics. Innovative applications are catalysis supports, high temperature resistant filters, biomedical implants etc. (Fan et al. 2005; Rambo et al. 2005; Singh and Yee 2004).

References

Anonymous (2006) Injection Moulding Compounding IMC. Krauss-Maffei Kunststofftechnik GmbH, München, Germany

Aspeborg H, Schrader J, Coutinho PM, Stam M, Kallas A, Djerbi S, Nilsson P, Denman S, Amini B, Sterky F, Master E, Sandberg G, Mellerowicz E, Sundberg B, Henrissat B, Teeri TT (2005) Carbohydrate-active enzymes involved in the secondary cell wall biogenesis in hybrid aspen. Plant Physiol. 137: 983-997

Awano T, Takabe K, Fujita M (2002) Xylan deposition on secondary wall of Fagus crenata fiber. Protoplasma 219: 106-115

Azizi Samir MAS, Chazeau L, Alloin F, Cavaille JY, Dufresne A, Sanchez JY (2005) POE-based nanocomposite polymer electrolytes reinforced with cellulose whiskers. Electrochimica Acta 50: 3897-3903

Bismarck A, Aranberri-Askargorta I, Springer J (2002) Surface characterization of Flax, Hemp and Cellulose Fibers; Surface Properties and the Water Uptake Behavior. Polymer Comp 23: 872-894

Boudet AM (1998) A new view of lignification. Trends Plant Sci 3: 67-71

Bouton S, Leboeuf E, Mouille G, Leydecker Marie T, Talbotec J, Granier F, Lahaye M, Hofte H, Truong Hoai N (2002) Quasimodo1 encodes a putative membrane-bound glycosyltransferase required for normal pectin synthesis and cell adhesion in Arabidopsis. Plant Cell 14: 2577-2590

Burgert I, Fruehmann K, Keckes J, Fratzl P, Stanzl Tschegg S (2004) Structure-function relationships of four compression wood types: micromechanical properties at the tissue and fibre level. Trees 18: 480-485

Carpita N, Tierney M, Campbell M (2001) Molecular biology of the plant cell wall: Searching for the genes that define structure, architecture and dynamics. Plant Mol Biol. 47: 1-5

Crônier D, Monties B, Chabbert B (2005) Structure and chemical composition of bast fibres isolated from developing hemp stems. J Agric Food Chem. 53: 8279-8289

Day A, Addi M, Kim, W, David H, Bert F, Mesnage P, Ronaldo C, Chabbert B, Neutelings G, Hawkins S (2005) ESTs from the fibre-bearing stem tissue of flax (linum usitatissimum L.): Expression analysis of sequences related to cell wall development. Plant Biol. 7: 23-32

Day A, Ruel K, Neutelings G, Crônier D, David H, Hawkins S, Chabbert B (2005) Lignification in the flax stem: evidence for an unusual lignin in bast fibres. Planta 222: 234-245

Dill-Langer G, Cruz-Hidalgo R, Kun, F, Moreno Y, Aicher S, Herrmann HJ (2003) Size dependency of tension strength in natural fibre composites. Physica A: Stat Mechan Appl. 325: 547-560

Emons AMC, Kieft, H (1994) Winding threads around plant cells: Applications of the geometrical model for microfibril deposition. Protoplasma. 180: 59-69

Faik A, Price Nicholas J, Raikhel Natasha V, Keegstra K (2002) An Arabidopsis gene encoding an alpha-xylosyltransferase involved in xyloglucan biosynthesis. Proc Natl Acad Sci USA 99: 7797-7802.

Fan T, Sun B, Gu J, Zhang D, Lau LWM (2005) Biomorphic Al2O3 fibers synthesized using cotton as bio-templates. Scripta Materialia 53: 893-897

Felby C, L.S. P, Nielsen BR (1997) Enhanced Auto Adhesion of Wood Fibers Using Phenol Oxidases. Holzforschung 51: 281-286

Gassan J, Bledzki AK (1999) Possibilities for improving the mechanical properties of jute/epoxy composites by alkali treatment of fibres. Comp Sci Technol. 59: 1303-1309

Gassan J, Bledzki AK (2000) Possibilities to Improve the Properties of Natural Fiber Reinforced Plastics by Fiber Modification - Jute Polypropylene Composites. Appl Comp Mat. 7: 373-385

Gassan J, Gutowski VS (2000) Effects of corona discharge and UV treatment on the properties of jute-fibre epoxy composites. Comp Sci Technol. 60: 2857-2863

George J, Sreekala MS, Thomas S (2001) A Review on Interface Modification and Characterization of Natural Fibre Reinforced Plastic Composites. Polymer Eng Sci. 41: 1471-1485

Gindl W, Keckes J (2005) All-cellulose nanocomposite. Polymer Comp. 46: 10221-10225

Girault R, His I, Andeme-Onzighi C, Driouich A, Morvan C (2000) Identification and partial characteristion of proteins and proteoglycans encrusting the secondary wall of flax fibres. Planta 211: 256-264

Gustavsson MT, Persson PV, Iversen T, Hult K, Martinelle M (2004) Polyester coating of cellulose fibre surfaces catalyzed by a cellulose-binding module-Candida Antarctica lipase B fusion protein. Biomacromolecules 5: 106-112

Gustavsson MT, Persson PV, Iversen T, Martinelle M, Hult K, Teeri TT, Brummer III H (2005) Modification of cellulose fiber surfaces by use of a lipase and a zyloglucan endotransglycosylase. Biomacromolecules 6: 196-203

Gutowski TG (1997) Advanced Composite Manufacturing. Wiley-Interscience, New York

Gutowski WS (1999) Physical model of interface and interphase performance in composite materials and bonded polymers. Die Angewandte Makromolekulare Chemie 272: 51-56

Harig H, Bäumer R, Gerardi H (1994) Wie zuverlässig läßt sich die Bündelfestigkeit von Baumwolle bestimmen? Melliand Textilberichte 75: 966-970

Hashem M, Hauser P, Smith B (2003) Reaction efficiency for cellulose cationization using 3-chloro-2-hydroxypropyl trimethyl ammonium chloride. Textile Res J. 73: 1017

Henning F, Ernst H, Brüssel R (2005) LFTs for automotive applications. Reinforced Plastics 49: 24-33

Kessler RW, Becker U, Kohler R, Goth B (1998) Steam-Explosion of Flax - A superior Technique for Upgrading Fibre Value. Biomass and Bioenergy 14 :237-249

Kim JK, Mai YW (1998) Engineered Interfaces in Fibre Reinforced Composites. Elsevier, Amsterdam

Kohler KM, Wedler M, Barthold P (1997) Technische Anwendung von Hanffasern. in Symposium-Proceedings 2. Biorohstoff Hanf. Nova-Institut, Hürth

Kohler R, Kessler RW (1999) Designing Natural Fibers for Advanced Materials. in: Proceedings of 5th International Conference on Wood fiber-Plastic Composites. Madison (WI), USA

Kohler R, Dück R, Ausperger B, Alex R (2003) A numeric model for the kinetics of water vapor sorption on cellulosic reinforcement fibers. Comp Interf 10: 255-276

Kohler R, Nebel K (2005) Fibrillation of Natural Fibres - Increasing the specific surface for high-performance composites. In: Rohstoffe FN (ed) Schriftenreihe "Nachwachsende Rohstoffe", vol. 27, Landwirtschaftsverlag, Münster

Kozlowski R, Wladyka-Przybylak M (2004) Fiber modification. In: Wallenberger FT, Weston NE (eds) Natural Fibers, Plastics and Composites Kluwer Academic Publishers, Boston

Kvien I, Tanem BS, Oksman K (2005) Characterization of cellulose whiskers and their nanocomposites by atomic force and electron microscopy. Biomacromolecules 6: 3160-3165

Lee SM (1993) Handbook of Composite Reinforcements. VCH, Weinheim

Lichtenthaler FW, Peters S (2004) Carbohydrates as green raw materials for the chemical industry. Comptes Rendus Chimie 7: 65-90

Liepman AH, Wilkerson CG, Keegstra K (2005) Expression of cellulose synthase-like (Csl) genes in insect cells reveals that CslA family members encode mannan synthases. Proc Natl Acad Sci USA 102: 2221-2226

Madson M, Dunand C, Li X, Verma R, Vanzin Gary F, Caplan J, Shoue Douglas A, Carpita Nicholas C, Reiter Wolf D (2003) The MUR3 gene of Arabidopsis encodes a xyloglucan galactosyltransferase that is evolutionarily related to animal exostosins. Plant Cell. 15: 1662-1670

Marsh G (2003) Next step for automotive materials. Materials Today 6: 36-43

Mathew AP, Dufresne A (2002) Morphological investigation of nanocomposites from sorbitol plasticized starch and tunicin whiskers. Biomacro molecules 3: 609-617

McQueen-Mason SJ, Cosgrove DJ (1994) Disruption of hydrogen bonding between plant cell wall polymers by proteins that induce cell wall extension. Proc Natl Acad Sci USA 91: 6574-6578

Mellerowicz EJ, Baucher M, Sundberg B, Boerjan W (2001) Unravelling cell wall formation in the woody dicot stem. Plant Mol Biol. 47: 239-274

Nebel K, Kohler R (2005) Fibrillation of natural fibres - Increasing the specific surface for high performance composites. In: Proceedings FAO/ ESCORENA International Conference Textiles for Sustainable Development. Port Elizabeth, South Africa

O'Donnell A, Dweib, MA, Wool RP (2004) Natural fiber composites with plant oil-based resin. Comp Sci Technol. 64: 1135-1145

Oksman KMS, Selin JF (2003) Natural fibres as reinforcement in polylactic acid (PLA) composites. Comp Sci Technol 63: 1317-1324

Ossola M, Galante YM (2004) Scouring of flax rove with the aid of enzymes. Enzyme Microb Technol. 34: 177-186

Pena MJ, Ryden P, Madson M, Smith AC, Carpita NC (2004) The galactose residues of xyloglucan are essential to maintain mechanical strength of the primary cell walls in Arabidopsis during growth. Plant Physiol. 134: 443-451

Peterson S, Jayaraman K, Bhattacharyya D (2002) Forming performance and biodegradability of woodfibre-Biopol composites. Composites Part A 33: 1123-1134

Pöhler E, Zimmermann T, Geiger T (2004) Cellulose-Nanofibrils for Polymer Reinforcement. in Global Wood and Natural Fibre Composites Symposium. Kassel, Germany

Poirier Y, Dennis DE, Klomparens K, Sommerville C (1992) Polyhydroxybutyrate, a biodegradable thermoplastic, produced in transgenic plants. Science 256: 520-523

Popovska N, Streitwieser DA, Xu C, Gerhard H (2005) Paper derived biomorphic porous titanium carbide and titanium oxide ceramics produced by chemical vapor infiltration. J Europ Ceram Soc. 25: 829-836

Pott GT (2004) Natural fibers with low moisture sensitivity in: Wallenberger FT and Weston NE (eds) Natural Fibers, Plastics and Composites Kluwer Academic Publishers, Boston

Prasad BM, Bhatnagar A, Sain MM (2003) High Performance Micro- and Nanobiofibrils Manufacturing Process. in 7th International Conference on Woodfibre-plastic Composites. Madison, WI

Rambo CR, Cao J, Rusina O, Sieber H (2005) Manufacturing of biomorphic (Si,Ti,Zr)-carbide ceramics by sol-gel processing. Carbon 43: 1174-1183

Reijmers TH, Maliepaard C, Van den Broeck H, Kessler RW, Toonen MAJ, Van der Voet H (2005) Integrated statistical analysis of cDNA microarray and NIR spectroscopic data applied to a hemp dataset. J Bioinf Comp Biol. 3: 891-913

Ridley BR, M.A., ON, Mohnen D (2001) Pectins: structure, biosynthesis, and oligogalacturonide-related signaling Phytochemistry 57: 929-967

Roberts AW, Roberts E (2004) Cellulose synthase (CesA) genes in algae and seedless plants. Cellulose 11: 419-435

Ryden P, Sugimoto-Shirasu K, Smith AC, Findlay K, Reiter WD, McCann MC (2003) Tensile properties of Arabidopsis cell walls depend on both a xyloglucan cross-linked network and rhamnogalacturonan II-borate complex. Plant Physiol. 132: 1033-1040

Sharma HSS, Faughey G, Lyons G (1999) Comparison of physical, chemical and thermal characteristics of water-, dew-, and enzyme-retted flax fibres. J Appl Polym Sci. 74: 139-143

Singh M, Yee BM (2004) Reactive processing of environmentally conscious, biomorphic ceramics from natural wood precursors. J Europ Ceram Soc. 24: 209-217

Snell KD, Peoples OP (2002) Polyhydroxyalkanoate polymers ant their production in transgenic plants. Metabol Engin. 4: 29-40

Sreekala MS, Thomas S (2003) Effect of surface modification on watersorption characteristics of oil palm fibres. Comp ScI Techn. 63: 861-869

Sterky F, Bhalerao RR, Unneberg P, Segerman B, Nilsson P, Brunner AM, Charbonnel Campaa L, Lindvall JJ, Tandre K, Strauss SH, Sundberg B, Gustafsson P, Uhlen M, Bhalerao RP, Nilsson O, Sandberg G, Karlsson J, Lundeberg J, Jansson S (2004) A Populus EST resource for plant functional genomics. Proc Natl Acad Sci USA. 101: 13951-13956

Stokes RJ, Evans DF (1997) Fundamentals of Interfacial Engineering. Wiley-VCH, New York

Thygesen LG, Hoffmeyer P (2005) Image analysis for the quantification of dislocations in hemp. Ind Crops Prod. 21: 173-184

Toonen MAJ, Maliepaard C, Reijmers TH, Van der Voet H, Mastebroek HD, Van den Broeck HC, Ebskamp MJM, Kessler W, Kessler RW (2004) Predicting the chemical composition of fibre and core fraction of hemp (Cannabis sativa L.). Euphytica 140: 39-45

Van den Broeck HC, Ebskamp MJM, Toonen MAJ, Koops AJ Differential expression of genes involved in C1 metabolism and lignin biosynthesis in wooden core and bast tissue of fiber hemp (Cannabis sativa L.). in preparation

Van Hattum FWJ, Nunes JP, Bernardo CA (2005) A theoretical and experimental study of new towpreg-based long fibre thermoplastic composites. Composites Part A: Appl Sci Manuf. 36: 25-32

Vanzin GF, Madson M, Carpita NC, Raikhel NV, Keegstra K, Reiter WD (2002) The mur2 mutant of Arabidopsis thaliana lacks fucosylated xyloglucan because of a lesion in fucosyltransferase AtFUT1. Proc Natl Acad Sci USA 99: 3340-3345

Wallenberger FT, Weston NE (eds) (2004) Natural Fibers, Plastics and Composites, Section III, natural Plastics & Matrix materials, Kluwer Academic Publishers, Boston

Widsten P (2002) Oxidative Activation of Wood Fibers for the Manufacture of Medium-Density Fiberboard (MDF). In: Series A15. Laboratory of Paper Technology, Helsinki University of Technology, Espoo

Willats WGT, McCartney L, Mackie W, Knox JP (2001) Pectin: cell biology and prospects for functional analysis. Plant Mol Biol. 47: 9-27

Wróbel M, Zebrowski J, Szopa J (2004) Polyhydroxybutyrate synthesis in transgenic flax. J Biotech. 107: 41-54

Yokoyama R, Nishitani K (2004) Genomic basis for cell-wall diversity in plants. A comparative approach to gene families in rice and Arabidopsis. Plant Cell Physiol. 45: 1111-1121

Yuan X, Jayaraman K, Bhattacharyya D (2004) Effects of plasma treatment in enhancing the performance of woodfibre-polypropylene composites. Composites: Part A 35: 1363-1374

Zafeiropoulos NE, Williams DR, Baillie CA, Matthews FL (2002) Engineering and characterisation of the interface in flax fibre/polypropylene composite materials. Part I. Development and investigation of surface treatments. Composites: Part A 33: 1083-1093

Zenoni S, Reale L, Tornielli GB, Lanfaloni L, Porceddu A, Ferrarini A, Moretti C, Zamboni A, Speghini A, Ferranti F, Pezzotti M (2004) Downregulation of the Petunia hybrida α-expansin gene PhEXP1 reduces the amount of crystalline cellulose in cell walls and leads to phenotypic changes in petal limbs. Plant Cell. 16: 295-308

7

In planta novel starch synthesis

Ravindra N. Chibbar, Seedhabadee Ganeshan, Monica Båga

Department of Plant Sciences, University of Saskatchewan,
51 Campus Drive, Saskatoon, Saskatchewan, Canada S7N 5A8
(e-mail: ravi.chibbar@usask.ca)

1. Introduction

Starch is the most abundant reserve carbohydrate present in higher plants, where it is predominantly found in the amyloplasts of storage organs such as roots, tubers and seeds. The green leaves have the unique ability to harvest light energy in the presence of carbon dioxide and water to synthesize carbohydrates, which are converted into starch. Starch present in the leaves is known as transitory starch, because it is broken down into simple sugars, which are transported to storage organs such as roots, tubers or seeds, where storage starch is synthesized for utilization by the plants at a later stage. Approximately 2050 and 679 million tonnes of storage starch is produced annually by the cereal and tuber crops, respectively, (Tester and Karkalas 2002) and harvested by humans for food, feed and industrial applications.

Starch is a staple in the diet of the world's population. Almost fifty percent of the calories in human diet are supplied by starch based products. In the tropics, the indigenous starch crops of the tropics, cassava, sorghum, millet or yam, are considered wonders of nature, because with the sun and rain, and little or no artificial inputs, they are able to grow in great abundance. For centuries, tropical starches have served as staple food for millions of people, throughout the hot and humid regions of the world. These starch crops are so proficient to supplying essential calories to even the

P. Ranalli (ed.), Improvement of Crop Plants for Industrial End Uses, 181–208.

very poorest people of the world that they are considered to be the quintessential subsistence crop.

In the developed countries, starch is also used in the food and beverage industries as a thickener and a sweetener, in a multitude of processed food products. Starch is one of the most preferred industrial raw materials and it is being used to produce hundreds of products for use in several industries such as paper and pulp, textiles, cosmetics, pharmaceutical, biodegradable plastic film, construction and mining. However, for industrial applications, native starches have to be chemically modified to confer the desired physical and chemical attributes (Ellis et al. 1998). The use of starch for industrial purposes will only become economically viable when its use as a raw material can compete with petroleum-based products. The renewable and biodegradable nature of starch makes it increasingly attractive in response to the environmental concerns about the industrial wastes generated from petroleum products and the growing awareness of the potential deleterious consequences of greenhouse gas emissions from these activities. However, if starch can be modified *in planta* to confer some of the desirable traits, which will reduce post-harvest processing and modification, the utility of starch as an industrial raw material will be significantly enhanced. Therefore, in the late twentieth century it was emphasized that the focus of starch production will shift from low value bulk starch to high value specialty starches (Stroh 1997). The non-food uses of starch are also a prime indicator of a country's economic condition. During periods of reduced economic growth, the volume of starch going into non-food use also drops considerably. On the other hand, during periods of rapid growth, the demand for construction materials for building industrial plants and housing, paper for packaging and wrapping various products, place a high demand on industrial raw materials (Jobling 2004).

The demand for industrial starches is increasing, but four major crops, potatoes, maize, wheat and tapioca supply most of the requirements for non-food industrial applications. Starch properties differ between the plant sources, but for specialized applications, the native starches are chemically modified. Therefore, it is advantageous to produce novel or tailor-made specialty starches *in planta* as it would decrease the currently imperative post-harvest modification, some of which are environmentally damaging (Slattery et al. 2000). Recent progress in the understanding of starch structure and biosynthesis and the development of molecular biology strategies to alter cellular metabolism has provided an opportunity to change starch structure *in planta*. In this chapter, we will discuss the storage starch structure, biosynthesis and strategies for its genetic modification for food, feed and industrial uses.

2. Starch structure

Starch is stored as crystalline or semi-crystalline water-insoluble granules with an internal lamellar structure, which consists of two types of glucan polymers, amylose and amylopectin, trace amounts of lipids and proteins (Buléon et al. 1998). Amylose is a predominantly linear glucan polymer, composed of α-1,4-linked glucose residues, that is sparsely branched with α-1,6-linkages (approximately one branch per 1,000 glucose residues) (Takeda et al. 1986; Takeda and Hizukuri 1987). The degree of polymerization (dp) of amylose molecules is species dependent, ranging from around 800 in maize and wheat to approximately 4500 in potato (Morrison and Karlakas 1990; Alexander 1995). Amylopectin on the other hand is a much larger (dp 10^5 to 10^7) and more complex glucan polymer. It is composed of hundreds of short α-1,4 glucan chains joined together by α-1,6-linkages, with approximately 5 % of the residues having both α-1,4 and α-1,6-linkages (Myers et al. 2000). Studies have shown that both the length of the glucan branch chains and their pattern of branching are non-random. The ordered nature of the α-1,6-linkages indicates that the branches in the amylopectin molecule are clustered. The branch length distributions of amylopectins from a variety of different plants were shown to be polymodal, with peaks at approximately dp 15, and dp 45 (Hizukuri 1985). The unique and highly-ordered structure of the amylopectin molecule, in terms of both branch location and branch length, is essential to the formation of the starch granule (Myers et al. 2000; Buléon et al. 1998). A normal starch granule is made up of one-quarter amylose and three-quarters amylopectin. The ratio of amylose to amylopectin is an important determinant of starch physical properties and its end-use and its alteration will be discussed in subsequent sections. Other components within all starch granules are proteins (0.5% in cereal endosperm and 0.05% in potato tuber), which include the enzymes of starch biosynthesis and may contribute to the flavor of starch, and lipids (1% in cereal endosperm and 0.1% in potato tuber).

The granular structure is relevant when considering the mechanism of starch degradation, as many glucan metabolizing enzymes appear to be unable to act upon intact granules as substrate (Buléon et al. 1998). Starch is accumulated in a diurnal fashion in chloroplast of leaves, and in photosynthetic competent cells (transitory starch) to store sugar produced from photosynthesis. Starch stored in this way is degraded at night (dark period) for energy source and growth. Starch is stored in tuberous tissues (roots) and seeds are also used for an energy source for the formation of the next plant generation. Transitory starch has small granules, while reserve starch granules have species specific size and shapes (Davis et al. 2003).

3. Starch biosynthesis

Studies on starch biosynthesis in plants have mainly been focused on pea, maize, rice, potato, wheat and barley where starch properties are important factors for crop quality. These studies have been aided by the availability of a large number of starch mutants primarily in pea, maize and barley, but also mutants created in the non-crop plant *Arabidopsis* and the unicellular green algae *Chlamydomonas*. Several recent reviews have summarized the extensive data related to starch biosynthesis (Ball and Morell 2003; Morell and Myers 2005; Smith 2001). Studies of starch mutants have so far produced a general consensus that ADP-glucose pyrophosphorylase (AGPase), soluble starch synthases (SS), starch branching enzymes (SBE), starch debranching enzymes (DBE; pullulanase; isoamylase) and possibly also disproportionating enzyme (D-enzyme) catalyze the final steps leading to amylopectin synthesis (Figure 7.1). Granule bound starch synthase 1 (GBSS 1) is the only enzyme committed to amylose synthesis. Several of the starch biosynthetic enzymes exist in different isoforms, some of which vary in their sub-cellular distribution, enzyme specificity, temporal activity and interaction with other enzymes, thus causing variation of the starch biosynthetic pathway in different plant species and tissues. A minimal subset of 14 conserved starch biosynthetic enzymes (two AGPases, five SS, three SBE and four DBE) is homologous in all the plant species studied to-date (Morell and Myers 2005). Besides the core enzymes, additional enzymes such as starch phosphorylases, disproportionating enzymes and glucan water kinases also play important roles in starch biosynthesis.

AGPase is the first enzyme committed to starch biosynthesis and catalyzes synthesis of ADP-Glu and pyrophosphate from ATP and Glu-1-P (Fig. 7.1). ADP-Glu is then used as glucose donor for synthesis of linear chains on amylose and amylopectin, the two glucan polymers accumulated in the water-insoluble starch granules. AGPase has long been considered a key regulatory enzyme in the starch pathway and is composed of two large and two small subunits, both affecting allosteric and catalytic properties of the enzyme (Cross et al. 2004; Hwang et al. 2005). AGPase is activated by 3-phosphoglyceric acid (3-PGA), a product of photosynthesis, and inhibited

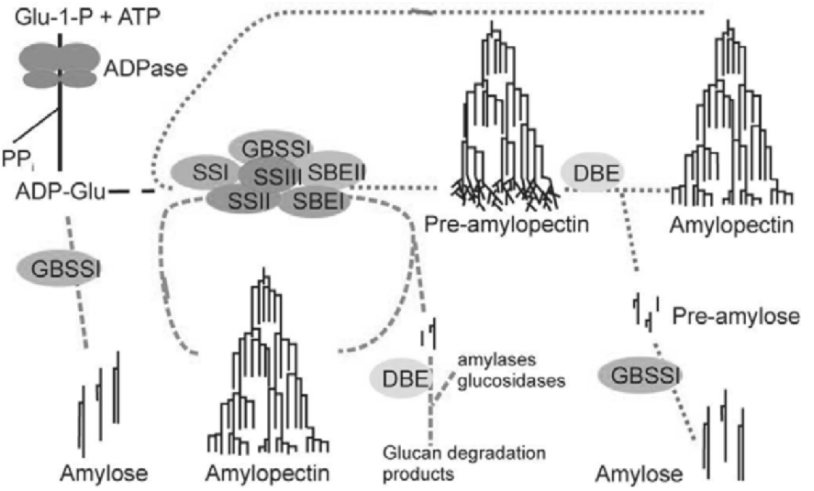


Fig. 7.1. Schematic illustration of two models for starch biosynthesis in plants. The "preamylopectin trimming model" is illustrated by the dark grey pathway and the "soluble glucan recycling model" is shown by the light grey pathway.

by inorganic phosphate (P_i) in many plant tissues (Preiss and Sivak 1988). In addition, some cereal AGPases are sensitive to heat stress, which affects crop yields (Singletary et al. 1994). AGPase is also under tight redox control in leaves, where the reducing conditions during the light cycle causes a thioredoxin-mediated reduction of a regulatory disulphide bond present between the two small subunits (Hendriks et al. 2003). The reduction of AGPase results in increased activity and higher activation by 3-PGA than obtained from the oxidized form present during the dark cycle. Due to redox and allosteric regulation of AGPase, the ADP-Glu production in green tissues is tightly coordinated with the day/night cycles and carbon status of the source tissue (Gibon et al. 2004).

In addition to ADP-Glu synthesized by the plastidial AGPase, an alternative source of ADP-Glu for starch biosynthesis in leaves has been proposed (Munoz et al. 2005). According to this study, a substantial amount of ADP-Glu is produced in the cytosol by the action of sucrose synthase (SuSy) and imported into the chloroplast for starch synthesis. In this alternative model, the role of plastidial ADPase is merely as a scavenger of breakdown products from starch degradation occurring during the dark cycle.

In developing grains of cereals, most of the AGPase activity is localized to the cytosol of the endosperm (Denyer et al. 1996b; Thorbjørnsen 1996; Beckles et al. 2001). This contrasts the location of AGPase in other cereal tissues and sink tissues of non-cereal plants where the AGPase activity is

exclusively found in plastids (Beckles et al. 2001). The presence of ADP-glucose transporter channels in the plastid membrane allows ADP-Glu formed in the cytosol of cereal endosperm to be imported into plastids and utilized for starch biosynthesis (Shannon et al. 1998; Patron et al. 2004). It has been speculated that the presence of a cytosolic, as well as, a plastidial AGPase in cereals will allow preferential channeling of available sucrose to starch biosynthesis at the expense of competing pathways utilizing sucrose-derived hexose phosphates as substrates (Beckles et al. 2001). In contrast to the leaf AGPases, the AGPases in sink tissues of different plant species show variable levels of allosteric regulation. In barley, wheat and maize, the endosperm AGPases are far less dependent on 3-PGA for activation than the leaf enzyme (Burger et al. 2003; Gómez-Casati and Iglesias 2002).

The main enzymes catalyzing synthesis of amylose and amylopectin from ADP-Glu are all located within plastids and include SS, SBE and DBE (Figure 7.1). It has long been believed that SS requires a primer to initiate glucan synthesis and it has been poorly understood how the polymerization reaction is primed. One hypothesis is that a protein with similar function as the self-glycosylating glycogenin, the initiator of glycogen biosynthesis in yeast and animal cells (Cheng et al. 1995), is involved in the priming reaction. The latter hypothesis is supported by RNAi experiments in *Arabidopsis* where down-regulation of a gene encoding a glycogenin-like protein in plastids resulted in reduced starch production (Chatterjee et al. 2004). A model that excludes the need of a primer to initiate glucan polymerization has also been put forward (Mukerjea and Robyt 2004). This model is based on growth of the glucan chain from the reducing end, as opposed to the long held belief that glucose units are added to the non-reducing end of the glucan.

The glucan chain elongation is catalyzed by four main SS: SSI, SSII, SSIII and granule bound SS (GBSSI). These enzymes catalyze formation of α-1,4-linkages between the glucose units resulting in linear glucan chains. SSI, SSII and SSIII are primarily involved in amylopectin biosynthesis, whereas GBSSI is the only enzyme associated with amylose production. The shortest chains on the amylopectin molecule are mainly synthesized by SSI and the longer chains are generally the result of SSII, SSIII and GBSSI actions. The relative contribution of each SS differs between plant species/tissues (Smith et al. 1997), and alterations to SS activities will affect the amylopectin fine-structure and/or starch granule morphology (Craig et al. 1998; Edwards et al. 1999; Morell et al. 2003).

The branches on the amylose and amylopectin molecules are introduced by SBE, which catalyzes cleavage of α-1,4 linkages and attaches the released chain through an α-1,6 bond to a new site on the glucan molecule. Two classes of SBE, SBEI and SBEII, with different substrate specificities

have been identified in plants. Long chain branches are introduced by SBEI activity, whereas SBEII prefer transfer of shorter chains (Preiss and Sivak 1998). A larger form of SBEI, SBEIc, has been identified in plants of the *Triticeae* tribe and is preferentially associated with the large A-type granules (Båga et al. 2000; Peng et al. 2000).

Debranching activities, exerted by isoamylase and pullulanase enzymes, have been postulated to have an active role in starch biosynthesis (Ball et al. 1996; Myers et al. 2000). The exact role of DBE in starch biosynthesis is not clear and two models for amylose and amylopectin biogenesis have emerged that includes different roles for DBE (Fig. 7.1). The "preamylopectin-trimming model" suggests that the outer branches of preamylopectin molecules are being trimmed by DBE to facilitate chain elongations by SS (Mouille et al. 1996). This will form amylopectin with an ordered branch structure and allow packaging of the molecule into starch granules. In addition, the glucan chains released by the DBE action on amylopectin can be elongated by GBSSI to form the amylose fraction. The role of DBE in the "soluble glucan recycling model" is to participate in degradation of short chain glucan molecules that may be produced by SS or SBE action to prevent accumulation of highly branched soluble polymers at the expense of amylopectin formation (Zeeman et al. 1998). Several studies have suggested that amylose is synthesized within the starch granules (Kuipers et al. 1994; Denyer et al. 1996a), whereas the much larger and highly branched amylopectin biosynthesis occurs at the surface of the granules (Smith et al. 1997). The spatial separation of amylose and amylopectin synthesis may explain why the two polymers with very different structures and sizes, can be produced simultaneously in plastids.

4. In planta starch modification strategies

The complexity of the starch biosynthetic pathway has been a challenge for the targeted *in planta* modification of starch to cater to novelty end uses for the food, health and industrial applications. Nonetheless, significant progress made towards better understanding of starch biosynthesis and altering biochemical pathways in plants has made possible to *in planta* alter the physico-chemical properties of starch. The availability of mutagenized populations, gene knockouts, over-expression and silencing approaches can all aid in gene function assessment and alteration. Therefore extensive efforts have been made to achieve similar end results within the plant itself. From screening for naturally occurring starch mutants to mutation breeding strategies to the use of newer technologies, including genetic transformation and genomics tools, the ability to alter starch

functionality has been demonstrated. A few strategies that have been successfully employed for *in planta* starch modification are described in the following sections.

4.1 Mutation breeding

To incorporate new desirable traits into their breeding programs, plant breeders screen for naturally occurring mutants from wild species of cultivated crops and land races. However, such screening procedures are sometimes time-consuming and labor-intensive and identification of the desired traits may often be challenging. Screening for starch mutants has, in the past, been restricted to the phenotypic aberrances exhibited by the seeds and characterized as the commonly used terms such as waxy, sugary, shrunken, brittle and so on (Kossmann and Lloyd 2000). Among the naturally occurring starch biosynthetic variants, over a century ago maize mutants, *sugary1* (*su1*) were identified (Correns 1901; cited in Jones 1924), which have been associated with sweet corn. It is now known that the *su1* mutation in maize is due to a deficiency in the debranching enzyme (DBE) of the isoamylase-type (James et al. 1995). The food industry has benefited tremendously from such naturally occurring mutants and subsequently others such as *sugary enhancer* and *shrunken2* (Young et al. 1997), which has further improved the sweetness of corn. However, most of the available cereal mutants are in diploid species like maize, rice and barley. Maize mutants for the *Waxy* locus have also been identified and grown for commercial purposes. The *waxy* maize starch has found uses as food stabilizers and thickeners, and emulsifiers for salad dressings (Jobling 2004). *Waxy* maize starch is also used in the manufacture of gummed tapes as remoistening adhesives, in adhesives and in the paper industry (Fergason 2001). As opposed to high amylopectin maize, the *sugary2* mutants have increased amylose up to 40% (Takeda and Preiss 1993) due to a lack of SSIIa activity (Zhang et al. 2004). In recent years high amylose starches have come to be recognized for their health benefits (Jobling 2004) as well as other industrial applications. In cereal species like wheat the identification of mutants is more difficult due to its hexaploid nature. Screening starch granule bound polypeptides useful natural starch mutants of wheat have been identified (Båga et al. 1999; Chibbar and Båga 2003). Naturally occurring starch mutants have not been found in potato (*Solanum tuberosum* L.) and other means of generating mutants by induced mutation, tissue culture and genetic transformation have been attempted. In the tropically grown cassava plant (*Manihot esculenta* Crantz) natural mutants for starch

have been identified with high free sugar content and lacking expression of the gene for starch branching enzyme (Carvalho et al. 2004).

Although screening for naturally occurring mutants has been and is still practiced, alternative ways to create variability are being explored. Even though induced mutations may eventually result in the release of varieties with altered traits, the resource-demanding nature of the process can preclude the benefits. This is because induced mutations, whether physical or chemical, are non-targeted leading to a large number of wasteful mutants. Induced mutants for starch biosynthesis have been generated in many plants. The model plant, *Arabidopsis thaliana*, is probably one of the best examples for induced mutation studies for starch biosynthesis, although it is not a starch storing plant. It has been suggested that study of transitory starch synthesis in leaves of mutant *Arabidopsis* may help to better understand the role of starch debranching enzymes in cereals (Zeeman et al. 1998). Due to the ease of inducing mutations and ease of genetically transforming *Arabidopsis*, it has been useful for studying starch biosynthesis and other metabolic pathways. Hovenkamp-Hermelink et al. (1987) induced mutations by X-ray irradiation to produce amylose-free starch in potato. In barley chemical mutagenesis was used to produce starch synthase IIa deficient lines with amylose content of 65-70% (Morrell et al. 2003) and one of the lines was shown to improve large bowel health in rats (Bird et al. 2004). In rice, retrotransposon-mediated insertion led to the production of mutants deficient for starch synthase I (SSI), wherein there was a reduction in amylopectin chain length (Fujita et al. 2006).

4.2 Genetic transformation

Among the starch storing plants, dicotyledonous species, such as potato, have been more amenable to genetic transformation as opposed to the monocotyledonous species such as the cereals. Potato has, therefore, served as a model system for genetic engineering of starch biosynthesis *in planta* using both the particle bombardment as well as *Agrobcaterium* species. In recent years, however, significant progress has also been made in the genetic transformation of cereals, primarily due to improved regeneration from cultured tissues and the ability to use *Agrobacterium tumefaciens*, which was earlier thought to be non-competent for the transformation of cereals. Since the first reports on the transformation of a Japonica rice (Hiei et al. 1994) and an Indica rice (Rashid et al. 1996) using *Agrobacterium*, there have been numerous reports on the *Agrobacterium*-mediated transformation of all the major cereals. Genetic transformation for *in planta* modification of starch was first successfully achieved in

potato, wherein an antisense construct for *GBSS1* gene was introduced using *Agrobacterium rhizogenes* to down-regulate expression of the endogenous *GBSSI* (Visser et al. 1991). Transformed potato lines exhibiting 70-100% reduction in GBSSI activity were produced, with reduced to amylose-free starch. The availability of efficient physical and biological DNA delivery systems for the transformation of dicotyledonous species has resulted in a number of reports of *in planta* starch alteration; albeit with varying success as to the extent the desired change occurred. For dicotyledonous species like potato, *Agrobacterium* is still the preferred method due to its efficiency. However, in spite of the perceived ease for *in vitro* culture and transformation of potato, not all varieties have been amenable to transformation and those that have been transformed have not always shown consistent expression levels of transgenes (Dale et al. 1995; Heeres et al. 2002). This genotype-dependent effect implied that tissue culture and transformation strategies had to be re-visited for poorly responsive genotypes and different regeneration and/or transformation protocols needed to be used for different genotypes. Heeres et al. (2002) attempted the transformation of 16 varieties of potato with an antisense construct for *GBSSI* and found variable degree of suppression of *GBSSI*, reduced yield and reduced starch content in some of the transformants compared to wild-type plants. The antisense inhibition of ADP-glucose pyrophosphorylase (AGPase) in potato resulted in reduced starch in tubers and accumulation of sucrose and glucose (Muller-Rober et al. 1992), while over-expression of an *E. coli* AGPase resulted in increase in sucrose to starch conversion (Stark et al. 1992). The transformation of other starch root crops has also been attempted. Starch crops such as cassava (*Manihot esculanta* Crantz) and sweet potato (*Ipomoea batatas*) are tropical habitat plants and consumed in many parts of the world. Raemakers et al. (2003) produced transgenic cassava plants with amylose free starch as a result of the integration of an antisense *GBSS* construct. Silencing of *GBSSI* gene in a transgenic sweet potato line led to the production of amylose free starch in tubers, in spite of the gene being introduced in sense orientation (Kimura et al. 2001).

For cereal transformation, the particle bombardment approach has been most widely used, prior to *Agrobacteirum* being shown to be transformation competent. There have been a number of obstacles for cereal transformation to become routine, particularly for the temperate cereals. First, the lack of efficient regeneration systems for cereals prevented production of high transformation frequencies of transformants. Furthermore, the tissue culture responses were found to be highly genotype-dependent and only a few model genotypes were consistently used for transformation experiments. The high yielding commercial varieties were generally less

responsive to tissue culture and were not targeted for transformation. Generally, immature embryos, immature scutella and inflorescences have been found to be responsive in tissue culture and competent for transformation. However, growth and maintenance of donor plants for isolation of immature explant tissues is very critical for high efficiency tissue culture response. Therefore the plants have to be grown under carefully controlled conditions and adequately fertilized to maintain their optimum physiological state and thereby improve tissue culture response. Consequently, growth of donor plants under such conditions adds to the cost, besides being time-consuming. Isolation of the immature tissues is also laborious and has to be done with minimal damage to the explants in order not to affect regeneration response. Furthermore the callus phase prior to plant regeneration has been known to be susceptible to somaclonal variation (Larkin and Scowcroft 1981). This occurs in monocotyledonous as well as dicotyledonous plants. This variation has been exploited for the regeneration and identification of useful variants and perceived, especially in the eighties as a novel source of *in vitro*-induced mutation. In combination with selection pressure in culture, regeneration of a number of plants with improved traits such as disease resistance and herbicide resistance have been obtained and released as varieties.

With the development of more specialized media, commercial varieties of the major temperate cereals are now amenable to high frequency regeneration in a fairly genotype independent manner using mature embryos (Ganeshan et al. 2006). The mature embryo has an added advantage since there is no requirement for the growth and maintenance of donor plants for explant isolation for *in vitro* culture. Mature embryos can be readily isolated from available mature seeds even from field-grown plants. The need for vernalization of winter cereals for obtaining immature explants for culture is also eliminated. The mature embryo culture is thus a simple, efficient and expedited approach for regeneration from cereals. The mature embryos have also been shown to be transformation competent using particle bombardment.

With the transformation methodology now being a routine process in most laboratories involved in transgenics, the focus has shifted from single gene transfer to studying and assessing multiple transgene functions. This becomes more relevant for the manipulation of the complex starch biosynthetic pathway, wherein multiple control points and interactions occur, including pleiotropic effects. To this end transfer of large inserts carrying genes involved in starch biosynthesis would be valuable for *in planta* starch modification. Recent advances in plant transformation technology using *Agrobacterium* and specialized vectors have led to the possibility of inserting large fragments of DNA into the plant genomes. In order to

achieve this, binary bacterial artificial chromosomes (BIBAC) vectors were developed for plant transformation (Hamilton et al. 1996; Hamilton 1997). More recently transformation–competent artificial chromosome (TAC) vectors (Liu et al. 1999) containing large-insert genomic DNA of hexaploid wheat has been developed (Liu et al. 2000). Using TAC vectors in *Agrobacterium*, transgenic rice carrying an 80 kb insert was recovered (Liu et al. 2002). Even though the stability of these large inserts has been questioned, progress has been made in determining factors influencing such instability and efficiency for transformation of tomato (Frary et al. 2001), potato (Song et al. 2003) and rice (Nakano et al. 2005). With the availability of such vectors for large inserts, it would be possible to use a multi-gene linking approach to place a number of the cloned starch biosynthetic genes in tandem and study their effects on *in planta* changes in starch. Using this approach, Lin et al. (2003) were able to stack multiple genes (including 10 genes and functional DNA elements) into a TAC vector and successfully transformed rice plants. This approach also offers the possibility of studying potential gene interactions by pyramiding only those starch biosynthetic genes suspected to be involved in the concerted metabolism of starch.

The available loss-of-function production systems such as induced mutations, insertional knock-out mutagenesis and antisense down-regulation have been effective to varying degrees in plants, and as mentioned earlier, *Arabidopsis thaliana* being the most versatile. But generally these approaches are time-consuming, unpredictable and desired alterations may not always occur in the more complex plant genomes such as wheat. The hexaploid wheat genome is made up of three different genomes - A, B and D genomes. Due to the occurrence of mostly three homoeologues of a particular gene in the wheat genome, there is a compensation effect from loss-of-function of one homoeologue. Therefore, studying the effect of a null allele on starch biosynthesis for example becomes complicated and confounded. More recently there has been interest in RNA interference (RNAi) as an approach at gene silencing. RNAi is a post-transcriptionally occurring gene silencing mechanism induced by double-stranded RNA and was first reported in the nematode worm, *Caenorhabditis elegans* (Fire et al. 1998). RNAi-mediated silencing in plants has also been shown (Waterhouse et al. 1998). Although RNAi is not a knock-out strategy per se, its down-regulatory potential can be specifically targeted to each gene of interest and would be valuable for polyploid species like wheat for both starch biosynthetic gene function analysis as well as for targeted *in planta* starch modification.

The application of the RNAi technology to plants is straightforward and relies on the availability of a transformation system and carefully designed transgene construct such that the transgenes produce hairpin RNA (hpRNA) with double-stranded RNA (dsRNA) regions (Waterhouse and Helliwell 2002). Several studies have explored the possibility of using the RNAi technology for reverse genetics or for *in planta* starch alterations using model plants. Using an RNAi approach in tobacco plants Chen et al. (2005) targeted the gene that codes for sucrose-6-phosphate phosphatase, which catalyzes the last step in the sucrose biosynthetic pathway converting sucrose-6-phosphate to sucrose. Transgenic tobacco lines were found to accumulate 3-5 times more starch than non-transformed plants. Silencing of β-amylase8 by RNAi in transgenic *Arabidopsis* plants led to starch accumulation in the leaves, with or without a six hour cold treatment at 4^{o}C (Kaplan and Guy, 2005). Induction of β-amylase occurs during temperature stresses and leads to increase in maltose content to protect membrane proteins and the electron transport chain (Kaplan and Guy 2004). A similar observation has been reported in potato tubers stored at low temperatures, wherein maltose accumulation occurred (Nielsen et al. 1997). Potato could therefore be an ideal candidate for RNAi-mediated silencing of β-amylase to increase starch content. More recently, Regina et al. (2006) produced high-amylose wheat by RNAi silencing of two starch branching enzyme II isoforms (SBEIIa and SBEIIb), leading to more than 70% amylose content. This resistant starch wheat was shown to improve large bowel indices in rats and offers potential for designing healthy starches for humans (Regina et al. 2006).

4.3 Tilling

As a retort to induced mutations, a targeted mutation strategy termed TILLING (Targeting Induced Local Lesions IN Genomes) was developed (McCallum et al. 2000). TILLING combines classical chemical mutation approach with high-throughput molecular biology techniques for the identification of desired mutants. It offers the possibility of identifying single base pair changes in a gene of interest (Till et al. 2003). The TILLING strategy involves mutagenizing seeds with ethlylmethane sulfonate (EMS), growing plants (M1), allowing selfing, planting of individual M2 seeds for DNA extraction from leaves and cataloguing M3 seeds. EMS primarily causes G/C to A/T transitions and in *Arabidopsis thaliana* up to 99.5% of mutations were shown to be randomly distributed G/C to A/T transitions (Greene et al. 2003). DNA extracted from the M2 plants is pooled for PCR of target gene. The PCR products are denatured and allowed to re-anneal

for the formation of heteroduplexes. In the original protocol, the heteroduplexes were analyzed for mutations on denaturing HPLC (McCallum et al. 2000). This was subsequently modified wherein the heteroduplexes were treated with an endonulcease, CELI, which cleaves mismatches in the heteroduplexes between mutated and non-mutated variants (Colbert et al. 2001). CELI, identified in celery, reconizes single base mismatches and cleaves on the 3'-side of the mismatch (Oleykowski et al. 1998). The cleaved heteroduplexes are separated by electrophoresis and mutations are identified. DNA from individual samples of the pool are then screened to identify the mutant plant. TILLING populations have been established for plant species such as *Arabidopsis thaliana* (Till et al. 2003), *Lotus japonica* (Perry et al. 2003), maize (Till et al. 2004), barley (Caldwell et al. 2004) and wheat (Slade et al. 2005). The wheat TILLING population is of significance since it has enabled the identification of a range of *waxy* phenotypes from close to normal to near-null (Slade et al. 2005).

5. Targets for in planta modification of starch synthesis

5.1 Increased starch quantity

Increased starch quantity in storage organs has several advantages therefore it was one of the first targets to successfully apply molecular techniques to alter starch biosynthesis. In the first instance, AGPase, the rate limiting enzyme in starch biosynthesis, was genetically manipulated to increase starch production and/or content in potato and maize. A 30% increase in starch content was observed in transgenic potato expressing an *E. coli* AGPase that is insensitive to P_i, a feed-back regulator of AGPase (Stark et al. 1992). Similarly, site-specific mutagenesis was used in maize to produce a mutant AGPase large subunit that was also insensitive to Pi. In this mutant, seed weight was increased 11-18% without increasing or decreasing the percentage of starch (Giroux et al. 1996). The mutant AGPase gene when introduced into wheat resulted in altered large AGP subunit which reduces P_i insensitivity and stable large and small subunit interactions (Smidansky et al. 2002). The transgenic wheat showed enhanced AGPase activity and 38% more seed yield and an increase in total plant biomass by 31% (Smidansky et al. 2002). These results are similar to those reported in maize (Giroux et al. 1996) and rice (Sakulsingharoj et al. 2004), where P_i insensitive AGPase large subunit gene did not increase grain starch concentration, but the seed weight was increased by 11-18%. Increased starch content in potato was also achieved through the manipulation

of a different enzyme, inorganic pyrophosphatase (PPi). Expression of an *E. coli* PPase in transgenic potato increased starch content by 20-30% (Geigenberger et al. 1998). Another approach to increase starch content in cereals is the manipulation of Bt-1, the plastidial adenylate transporter (Shannon et al. 1998). Bt-1 mutants in maize have markedly reduced starch contents (Creech 1965), and thus over-expression of Bt-1 may lead to increased starch content.

The increase of total starch concentration is not an economically viable alteration in starch metabolism. However, in some cases augmentation of starch synthesis may be needed to make the production of unique starches or other traits economically viable. Naturally occurring mutants and/or transgenic plants with altered starch biosynthesis often have significantly reduced starch contents associated with qualitative changes to the starch (Marshall et al. 1996; Creech 1965; Nishi et al. 2001). Therefore, increasing the starch content in mutants/transgenics may be necessary to increase the yields of the starch with altered structure.

5.2 Phosphorylated cereal starches

Phosphorous is present in minor quantities, but the relatively higher quantity and its chemical nature in tuber starch contributes to their superior functional qualities over cereal starch for several applications (Alexander 1995). Using the *in planta* modification strategies described in this chapter, it may be possible to produce phosphorylated starches in wheat, barley and maize with endosperm-specific expression of the R1 protein (Lorberth et al. 1998). Down regulating the R1 protein gene expression in potato and the concomitant reduction in starch phosphate content resulted in starch with reduced peak viscosity and elevated setback viscosity (Albertsson and Karlsson 1995). Therefore, transgenic expression of R1 protein in cereal grains will likely increase peak viscosity and reduce setback, in phosphorylated cereal starches. Such changes could enhance the utility of cereal starches for industrial purposes. However, the precise changes due to the phosphorylated cereal starch cannot be predicted as there are other differences between cereal and potato starch, such as the structure and content of amylose molecules, phospholipids content and granule size.

5.3 High phytoglycogen starches

The *sugary-1* mutants accumulate phytoglycogen, a highly-branched, water-soluble polysaccharide, in addition to starch in maize (Creech 1965) and rice (Nakamura et al. 1997), and the *Sta7* mutant in *Chlamydomonas* (Mouille et al. 1996). In both the maize and rice mutants, the phytoglycogen content is approximately 25-30% of total carbohydrates. Increasing the phytoglycogen content of maize to more than 30% could result in a storage polysaccharide with reduced viscosity, gel formation and retrogradation rate with increased water holding capacity (Johnson et al. 2001a). In addition, a grain with high phytoglycogen-starch combination would have increased digestibility as a livestock feed, which could potentially have an enormous economic impact (Johnson et al. 2001a,b). Attempts have been made to increase phytoglycogen concentration by stacking different mutant alleles in maize but have been unable to markedly increase phytoglycogen concentration.

The naturally occurring phytoglycogen accumulating sugary-1 mutants are often associated with a reduction in dry seed weight (Creech 1965). Increasing starch quantity through genetic engineering, as discussed in the previous section, may be necessary for the development of plants with a high phytoglycogen phenotype. In addition, the physiological role of the starch granule as a non-hygroscopic, non-osmotic energy store must also be considered. Higher phytoglycogen content may result in reduced starch concentration, and this could cause problems with germination and therefore agronomic viability. Thus, there may be a limit to which phytoglycogen content may be increased, without interfering with the physiological characters of the grain.

5.4 Starch with reduced amylose concentration

Amylose free (waxy) starch has unique properties and it is used in various industries such as textiles, corrugating and adhesive industries. These result from their clear film forming properties. Moreover, chemical modification of waxy starch further extends its industrial applications and an enormous array of applications in the food industry (Fergason 2001). Natural mutants lacking GBSSI activity produce starch granules containing only the amylopectin glucan polymer. The *waxy* mutants have been shown to be present in maize, rice and barley, but till recently had not been identified in crops with multiple genomes, such as wheat. During the last decade, through breeding it has been possible to recombine the three null GBSSI present in different wheat germplasms to develop lines with <1%

amylose (Nakamura et al. 1995; see recent reviews Seib 2000; Chibbar and Chakraborty 2004). "Partially *waxy*" starch, i.e. starch with amylose levels between normal and *waxy* starch, was identified as a potentially valuable quality trait in maize (Johnson et al. 2001a) and in wheat it is used for Asian noodle production (Hoshino et al. 2000; Yamaguchi et al. 2000). In wheat, the GBSS1 produced by the three genomes A, B and D, show activity levels in the order B>D>A (Miura and Sugiwara 1996) and therefore different combinations result in lines with varying amylopectin concentrations, known as 'partially waxy' starch. Partially waxy starch, i.e. starch with amylose levels between normal (25%) and *waxy* starch is desired for production of certain foods, e.g. Japanese noodles (Seib 2000). Partially waxy wheat starch has been obtained through breeding of germplasm carrying one or two GBSSI null alleles (Demeke et al. 1999). The partially waxy wheat starches contain about 12% amylose and have higher swelling power and show crystallization patterns and gelatinization properties distinct from both normal and waxy starch (Seib 2000; Chibbar and Chakraborty 2004).

5.5 Starches with high amylose concentration

Starch with elevated amylose concentration (≥ 40%) is preferred for gums and candies and in the paper and pulp industry. Recently, an increased demand for high amylose wheat starch within the food industry has emerged as high-amylose starch can be converted to resistant starch (RS) upon heating and subsequent cooling. RS is not digested in the small intestine, but is broken down by the bacteria in the colon. As a result, RS acts as dietary fiber (DF), reduces the calories from food, has low glycemic index and is considered beneficial for colon health. An increase in amylose/amylopectin ratio and higher average amylopectin branch length in starch is found in certain maize, rice and pea mutants, which lack expression of one of the two SBEII isoforms (Chibbar and Baga 2003). The high amylose mutants in maize and pea have amylose concentrations of 50% or greater as compared to 25-30% for the normal endosperm and embryo starches. In mutant indica rice, the amylose content is 39-41% compared to 24-25% for the wild type (Kaushik and Khush 1991). High amylose barley lines with grain endosperm amylose concentration up to 40% have been developed (B.G. Rossnagel, personal communication). The amylose fraction in some mutants has a lower molecular weight and wider size distribution than normal amylose. The amylopectin fractions of these starches are also more linear with longer branch length than normal starch. In high amylose barley (Himalaya) the mutation has been attributed to SSIIa deficiency (Morell et al. 2003). Similarly, elimination of SSII polypeptides in

wheat increased apparent amylose (colorimetric assays) concentrations to 30-37% (Yamamori et al. 2000). In a similar approach, wheat lines deficient in SSII A and B genome polypeptides, resulted in lines showing up to 35% amylose as determined by HP-SEC analyses (Chibbar et al. 2005). However, in rice, barley and wheat amylose concentrations could not be increased beyond 40%, although a recent report (Regina et al 2006) suggest that RNAi silencing of SBEIIb genes in primary wheat transgenics amylose concentrations can be increased to 70%.

5.6 Large or small granule starches

Starch granule size is an important factor in several industrial processes including the production of thin films (Lim et al. 1992), paper coatings, cosmetic products (Ellis et al. 1998), and carbonless copy paper (Nachtergaele and Van Nuffel 1989). Furthermore, starch granule size is important in the brewing process. A significant portion of the small B-type granules from barley is not completely gelatinized in the mash and the undegraded residue causes mechanical problems during subsequent processing, e.g. filtration (Tillett and Bryce 1993). There are significant additional processing costs required for the isolation of large or small granules. Thus, the *in planta* production of starches with predominantly large or small granules would be very desirable.

Very little is known about the processes determining starch granule size. A few studies have indicated that SBEI may be involved. Studies using antisense SBEI constructs have reported increased granule size in transgenic potato (Flipse et al. 1996). It has also been suggested from studies in wheat that an SBEI isoform, SBEIc, may be involved in determining bimodal starch granule size distribution in this crop. Production of the two size fractions of starch granules is only found in plants belonging to the Triticeae tribe, including wheat, barley rye and triticale. Analysis of wheat starch granule proteins has revealed a difference in the abundance of SBEIc, an isoform of starch branching enzyme, in small and large granules (Båga et al. 2000; Peng et al. 2000). SBEIc is a large (~150 kD) protein that, in contrast to the 87 kD SBEI, is preferentially incorporated into the large starch granules. SBEIc is only found in wheat, barley and rye starch granules, and thus, is associated with plants showing bimodal starch granule size distribution in the endosperm. Down-regulation or a mutation in either SBEI or SBEIc can alter the proportion of A- and B-type granules. Both transgenic-antisense SBEI and/or SBEIc approach or traditional plant breeding techniques with mutagenesis could be taken to develop starches with altered starch granule size.

5.7 Starches with altered chain lengths and branching patterns

The molecular structures of amylose, amylopectin and the starch granule are important determinants of the functional properties of a starch. There is potential to produce a wide range of new starches through the alteration of the glucan chain lengths, branching patterns and granule crystallinity (Johnson et al. 2001a). Mutations in several different starch synthetic enzymes have been shown to affect the branch length and degree of branching of amylopectin (Craig et al. 1998; Yamamori et al. 2000; Edwards et al. 1999) resulting in altered starch physicochemical properties (Yamamori et al. 2000; Edwards et al. 1999). Recently, transgenic expression of a bacterial glycogen branching enzyme gene in rice resulted in a marked increase in the degree of branching of amylopectin with high number of short branches (Kim et al. 2005). Given the many different enzymes involved in amylopectin biosynthesis, various modifications to amylopectin structure and a diverse range of concomitant functional changes are possible. Some functional changes in maize, including improved gel formation and stabilized viscosity have been postulated to have a significant economic value in maize (Johnson et al. 2001a).

6. Concluding remarks

Starch is one of the most abundant and versatile storage carbohydrates, which has a multitude of applications. Several modifications of starch structure have been made which have further enhanced its utility. Recent advances in our understanding of plant processes and cellular metabolism present numerous opportunities to modify starch structure for the benefit of humans. For example, the recent expression of the cellulose synthase-like (*CslF*) genes from rice in *Arabidopsis* resulted in the synthesis of (1,3:1,4)-β-D-glucan like polymers (Burton et al. 2006), which demonstrates that novel glucan polymers can be synthesized in plants. Maize starch with beta glucan linkages has been suggested as a potentially valuable trait in maize (Johnson et al. 2001a). Similarly, introduction of 1,3 and 1,6 linked glucosyl residues catalyzed by the transgenic expression of bacterial glucansucrases is another approach to modify glucan biosynthesis in plants (Kok-Jacon et al. 2003). The use of starch granules as carriers of valuable therapeutic drugs or other useful compounds has not been fully explored. Some of the starch biosynthetic enzymes are completely granule bound and this unique feature may be used to deposit compounds of interest in the starch granules. The hydrophobic nature of starch granules will facilitate purification of the compound of interest. New approaches to

study grain development and starch biosynthesis (Jansson et al. 2006) will further improve our understanding of starch biosynthesis which can be used to produce genetically modified starches *in planta*.

Acknowledgements

Canada Research Chairs, Canada Foundation for Innovation, National Science and Engineering Research Council, Saskatchewan Agricultural Development Fund, Genome Canada/Genome Prairie, Brewing and Malting Barley Research Institute and Saskatchewan Department of Industry are gratefully acknowledged for supporting research in our laboratories.

References

Albertsson A-C, Karlsson S (1995) Degradable polymers of the future. Acta Polym 46:114-123

Alexander RJ (1995) Starch in plastics. Cereal Foods World 41:426-427

Båga M, Nair RB, Repellin A, Scoles GJ, Chibbar RN (2000) Isolation of a cDNA encoding a granule-bound 152-kilodalton starch-branching enzyme in wheat. Plant Physiol 124:253-263

Båga M, Repellin A, Demeke T, Caswell K, Leung N, Abdel-Aal ES, Hucl P, Chibbar RN (1999) Wheat starch modification through biotechnology. Starch-Stärke 51:111-116

Ball SG, Morell MK (2003) From bacterial glycogen to starch: Understanding the biogenesis of the plant starch granule. Annu Rev Plant Biol 54:207-233

Ball SG, Guan H-P, James M, Myers A, Keeling P, Mouille G, Buléon A, Colonna P, Preiss J (1996) From glycogen to amylopectin: A model for the biogenesis of the plant starch granule. Cell 86:349-352

Ball SG, van de Wal MHBJ, Visser RGF (1998) Progress in understanding the biosynthesis of amylose. Trends Plant Sci 3:462-467

Beckles DM, Smith AM, Rees T (2001) A cytosolic ADP-glucose pyrophosphorylase is a feature of graminaceous endosperms, but not of other starch storing organs. Plant Physiol 125:818-827

Bird AR, Flory C, Davies DA, Usher S, Topping DL (2004) A novel barley cultivar (Himalaya 292) with a specific gene mutation in starch synthase IIa raises large bowel starch and short-chain fatty acids in rats. J Nutr 134: 831-835

Buléon A, Ball S, Planchot V, Colonna P (1998) Starch granules: structure and biosynthesis. Int J Biol Macromol 23: 85-112

Burger BT, Cross JM, Shaw JR, Caren JR, Greene TW, Okita TW, Hannah LC (2003) Relative turnover numbers of maize endosperm and potato tuber ADP-glucose pyrophosphorylases in the absence and presence of 3-phosphoglyceric acid. Planta 217: 449-456

Burton RA, Wilson SM, Hrmova M, Harvey AJ, Shirley NJ, Medhurst A, Stone BA, Newbigin EJ, Bacic A, Fincher GB (2006) Cellulose synthase-like *CslF* genes mediate the synthesis of cell wall (1:3;1:4)-β-D-glucans. Science 311: 1940-1942

Caldwell DG, McCallum N, Shaw P, Muehlbauer GJ, Marshall DF, Waugh R (2004) A structured mutant population for forward and reverse genetics in barley (*Hordeum vulgare* L.). Plant J 40: 143-150

Carvalho LJCB, de Souza CRB, Cascardo CM, Junior CB, Campos L (2004) Identification and characterization of a novel cassava (*Manihot esculenta* Crantz) clone with high free sugar content and novel starch. Plant Mol Biol 56:643-659

Chatterjee M, Berbezy P, Vyas D, Coates S, Barsby T (2004) Reduced expression of a protein homologous to glycogenin leads to reduction of starch content in *Arabidopsis* leaves. Plant Sci 168: 501-509

Chen S, Hajirezaei M, Peisker M, Tschiersch H, Sonnewald U, Börnke F (2005) Decreased sucrose-6-phosphate phosphatase level in transgenic tobacco inhibits its photosynthesis, alters carbohydrate partitioning, and reduces growth. Planta 221: 479-492

Cheng C, Mu J, Farkas I, Huang D, Goebl MG, Roach PJ (1995) Requirement of the self-glucosylating initiator proteins Glg1p and Glg2p for glycogen accumulation in *Saccharomyces crevisiae*. Mol Cell Biol 15: 6632-6640

Chibbar RN, Båga M (2003) Genetic modification of primary metabolism- carbohydrates. In: Thomas B, Murphy DJ, Murray BJ (eds) Encyclopedia of Applied Plant Sciences. Elsevier, Oxford, U.K. pp 449-459

Chibbar RN, Båga M, Ganeshan S, Hucl PJ, Limin A, Perron C, Ratnayaka I, Kapoor A, Verma V, Fowler DB (2005) Carbohydrate Modification to Add Value to Wheat Grain. In: Chung OK, Lockhart GL (eds), International Wheat Quality Conference Proceedings, Grain Industry Alliance, Manhattan, Kansas, USA pp 75-84

Chibbar RN, Chakraborty M (2005) Waxy wheat. In: Abdel-Aaal E, Wood P (eds) Specialty grains for food and feed. American Association of Cereal Chemists, Inc., St Paul, Minnesota, USA, pp 143-165

Colbert T, Till BJ, Tompa R, Reynolds S, Steine MN, Yeung AT, McCallum CM, Comai L, Henikoff S (2001) High-throughput screening for induced point mutations. Plant Physiol 126: 480-484

Craig J, Lloyd JR, Tomlinson K, Barber L, Edwards A, Wang TL, Martin C, Hedley CL, Smith AM (1998) Mutations in the gene encoding starch synthase II profoundly alter amylopectin structure in pea embryos. Plant Cell 10:413-426

Creech RG (1965) Genetic control of carbohydrate synthesis in maize. Genetics 52:1175-1186

Cross JM, Clancy M, Shaw JR, Greene TW, Schmidt RR, Okita TW, Hannah LC (2004) Both subunits of ADP-glucose pyrophosphorylase are regulatory. Plant Physiol 135:137-144

Dale PJ, Hampson KK (1995) An assessment of morphogenic and transformation efficiency in a range of varieties of potato (*Solanum tuberosum* L.). Euphytica 85: 101-108

Davis JP, Netrphan S, Khandelwal RL, Chibbar RN (2003) Synthesis of novel starches in planta: Opportunities and challenges. Starch-Stärke 55:107-120

Demeke T, Hucl P, Abdel-Aal ESM, Båga M, Chibbar RN(1999) Biochemical characterization of the wheat waxy A protein and its effect on starch properties. Cereal Chem 76:694-698

Denyer K, Clarke B, Hylton C, Tatge H, Smith AM (1996a) The elongation of amylose and amylopectin chains in isolated starch granules Plant J 10:1135-1143

Denyer K, Dunlap F, Thorbjornsen T, Keeling P, Smith AM (1996b) The major form of ADP-glucose pyrophosphorylase in maize endosperm is extra plastidial. Plant Physiol 112:779-785

Edwards A, Fulton DC, HYlton CM, Jobling SA, Gidley M, Rossner U, Martin C, Smith AM (1999) A combined reduction in activity of starch synthase II and III of potato has novel effects on the starch of tubers. Plant J 17:251-261

Ellis RP, Cochrane MP, Dale MFB, Duffus CM, Lynn A, Morrison IM, Prentice RDM, Swanston JS, Tiller SA (1998) Starch production and industrial use. J Sci Food Agric 77:289-311

Fergason V (2001) High amylose and waxy corns. In: Hallauer (ed) Specialty corns. CRC Press, Florida, USA, pp 63-84

Fire A, Xu SQ, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391:806-811

Flipse E, Suurs L, Keetels CJAM, Kossman J, Jacobsen E, Visser RGF (1996) Introduction of sense and antisense cDNA for branching enzyme in the amylose-free potato mutant leads to physicochemical changes in the starch. Planta 198:340-347

Frary A, Hamilton CM (2001) Efficiency and stability of high molecular weight DNA transformation: an analysis in tomato. Transgenic Res 10:121-132

Fujita N, Yoshida M, Asakura N, Ohdan T, Miyao A, Hirochika H, Nakamura Y (2006) Function and characterization of starch synthase I using mutants in rice. Plant Physiol 140:1070-1084

Ganeshan S, Chodaparambil S, Båga M, Fowler DB, Hucl P, Rossnagel BG, Chibbar RN (2006) In vitro regeneration for cereals based on multiple shoot induction from mature embryos in response to thidiazuron. Plant Cell Tiss Org Cult 85:63-73

Geigenberger P, Hajirezaei M, Geiger M, Deiting U, Sonnewald U, Stitt M (1998) Overexpression of pyrophosphorylase leads to increased sucrose degradation and starch synthesis, increased activities of enzymes for sucrose-starch interconversion, and increased levels of nucleotides in growing potato tubers. Planta 205:428-437

Gibon Y, Blasing OE, Palacios-Rojas N, Pankovic D, Hendriks JHM, Fisahn J, Hohne M, Gunther M, Stitt M (2004) Adjustment of diurnal starch turnover to short days: depletion of sugar during the night leads to a temporary inhibition of carbohydrate utilization, accumulation of sugars post-translational activation of ADP-glucose pyrophosphorylase in the following light period. Plant J 39:847-862

Giroux MJ, Shaw J, Barry G, Cobb BG, Greene T, Okita T, Hannah LC (1996) A single gene mutation that increases maize seed weight. Proc Natl Acad Sci USA 93:5824-5829

Gomez-Casati DF, Iglesias AA (2002) ADP-glucose pyrophosphorylase from wheat endosperm. Purification and characterization of an enzyme with novel regulatory properties. Planta 214:428-434

Greene EA, Codomo CA, Taylor NE, Henikoff JG, Till BJ, Reynolds SH, Enns LC, Burtner C, Johnson JE, Odden AR, Comai L, Henikoff S (2003) Spectrum of chemically induced mutations from a large-scale reverse-genetic screen in Arabidopsis. Genetics 164:731-740

Hamilton CM (1997) A binary–BAC system for plant transformation with high molecular weight DNA. Genetics 200:107-116

Hamilton CM, Frary A, Lewis C, Tanksley SD (1996) Stable transfer of intact high molecular weight DNA into plant chromosomes. Proc Natl Acad Sci USA 93:9975-9979

Heeres P, Schippers-Rozenboom M, Jacobsen E, Visser RGF (2002) Transformation of a large number of potato varieties: genotype-dependent variation in efficiency and somaclonal variability. Euphytica 124:13-22

Hendriks JHM, Kolbe A, Gibon Y, Stitt M, Geigenberger P (2003) ADP-glucose pyrophosphorylase is activated by posttranslational redox-modification in response to light and to sugars in leaves of Arabidopsis and other plants. Plant Physiol 133:838-849

Hermansson A-M, Svegmark K (1996) Developments in the understanding of starch functionality. Trends Food Sci 7:345-353

Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient Transformation of Rice (*Oryza sativa* L) Mediated by *Agrobacterium* and Sequence-Analysis of the Boundaries of the T-Dna. Plant J 6:271-282

Hizukuri S (1985) Relationship between the distribution of chain length of amylopectin and crystalline structure of starch granules. Carbohyd Res 141:295-306

Hoshino T, Yoshikawa R, Ito S, Hatta K, Nakamura T, Yamamori M, Hayakawa K, Tanaka K, Akashi H, Endo S, Tago S, Ishigami S (2000) Flour blends for breads, cakes, or noodles, and foods prepared from flour blends. US patent 6,042,867

Hovenkamp-Hermelink JHM, Jacobsen E, Ponstein AS, Visser RGF, Vos-Scheperkeuter GH, Bijmolt EW, de Vries JN, Witholt B, Feenstra WJ (1987) Isolation of an amylose-free starch mutant of the potato (*Solanum tuberosum* L.). Theor Appl Genet 75:217-221

Hwang S-K, Salamone PR, Okita TW (2005) Allosteric regulation of the higher plant ADP-glucose pyrophosphorylase is a product of synergy between the two subunits. FEBS Letters 579:983-990

James MG, Robertson DS, Myers AM (1995) Characterization of the maize gene *sugary1*, a determinant of starch composition in kernels. Plant Cell 7:417-429

Jansson C, Sun C, Ganeshan S, Chibbar RN (2006) Antisense ODN Inhibition as a Diagnsotic Tool in Studies on Cereal Grain Development. In: Esser K et al. (ed) Progress in Botany, 68, Springer Verlag, Berlin, pp 179-190

Jobling S (2004) Improving starch for food and industrial applications. Curr Opinion Plant Biol 7:210-218
Johnson LA, Hardy CL, Baumel CP, White PJ (2001a) Identifying valuable corn quality traits for starch production. Cereal Foods World 46:417-423
Johnson LA, Hardy CL, Baumel CP, Yu T-H, Sell JL (2001b) Identifying valuable corn quality traits for livestock feed. Cereal Foods World 46:472-481
Jones DF (1924) Selective fertilization among the gametes from the same indiviuals. Proc Natl Acad Sci 10:218-221
Kaplan F, Guy CL (2005) RNA interference of Arabidopsis beta-amylase8 prevents maltose accumulation upon cold shock and increases sensitivity of PSII photochemical efficiency to freezing stress. Plant J 44:730-743
Kaplan F, Guy CL (2004) b-Amylase induction and the protective role of maltose during temperature shock. Plant Physiol135:1674-1684
Kaushik RP, Khush GS (1991) Endosperm mutants in rice-gene expression in Japonicaand Indica backgrounds. Cereal Chem 68:487-491
Kim WS, Kim J, Krishnan HB, Nahm BH (2005) Expression of Escherichia coli branching enzyme in caryopses of transgenic rice results in amylopectin with an increased degree of branching. Planta 220:689-695
Kimura T, Otani M, Noda T, Ideta O, Shimada T, Saito A (2001) Absence of amylose in sweet potato [*Ipomoea batatas* (L.) Lam.] following the introduction of granule-bound starch synthase I cDNA. Plant Cell Rep 20:663-666
Kok-Jacon GA, Ji Q, Vincken J-P, Visser RGF (2003) Towards a more versatile α-glucan biosynthesis in plants. J Plant Physiol 160:765-777
Kossmann J, Lloyd J (2000) Understanding and influencing starch biochemistry. Crit Rev Plant Sci 19:171-226
Kuipers AGJ, Jacobsen E, Visser RGF (1994) Formation and deposition of amylose in the potato tuber starch granule are affected by the reduction of granule-bound starch synthase gene expression. Plant Cell 6:43-52
Larkin PJ, Scowcroft WR (1981) Somaclonal variation – a novel source of variability from cell cultures for plant improvement. Theor Appl Genet 60:197-214
Lim ST, Jane J, Rajagopalan S, Seib PA (1992) Effect of starch granule size on physical properties of starch-filled polyethylene film. Biotechnol Prog 8:51-57
Lin P-Y, Czuchajowska Z (1998) Role of phosphorous in viscosity, gelatinization and retrogradation of starch. Cereal Chem 75:705-709
Lin L, Liu Y-G, Xu X , Li B (2003) Efficient linking and transfer of multiple genes by a multigene assembly and transformation vector system. Proc Natl Acad Sci USA 100:5962-5967
Liu YG, Liu H, Chen L, Qiu W, Zhang Q, Wu H, Yang C, Su J, Wang Z, Tian D, Mei M (2002) Development of new transformation-competent artificial chromosome vectors and rice genomic libraries for efficient gene cloning. Gene 282: 247-255
Liu YG, Nagaki K, Fujita M, Kawaura K, Uozumi M, Ogihara Y (2000) Development of an efficient maintenance and screening system for large-insert genomic DNA libraries of hexaploid wheat in a transformation-competent artificial chromosome (TAC) vector. Plant J 23:687-695

Liu YG, Shirano Y, Fukaki H, Yanai Y, Tasaka M, Tabata S, Shibata D (1999) Complementation of plant mutants with large genomic DNA fragments by a transformation-competent artificial chromosome vector accelerates positional cloning. Proc Natl Acad Sci USA 96:6535-6540

Lorberth R, Ritte G, Willmitzer L, Kossman J (1998) Inhibition of a starch granule-bound protein leads to modified starch and repression of cold sweetening. Nat Biotech 16:473-477

Marshall J, Sidebottom C, Debet M, Martin C, Smith AM, Edwards A (1996) Identification of the major starch synthase in the soluble fraction of potato tubers. Plant Cell 8:1121-1135

McCallum CM, Comai L, Greene EA, Henikoff S (2000) Targeted screening for induced mutations. Nat Biotech 18:455-457

Miura H, Sugiwara A (1996) Dosage effects of three *Wx* genes on amylose synthesis in wheat endosperm. Theor Appl Genet 93:1066-1070

Morell MK, Myers AM (2005) Towards the rational design of cereal starches. Curr Opinion Plant Biol 8:204-210

Morell MK, Kosar-Hashemi B, Cmiel M, Samuel MS, Chandler P, Rahman S, Buleon A, Batey IL, Li Z (2003) Barley sex6 mutants lack starch synthase IIa activity and contain a starch with novel properties. Plant J 34:173-185

Morrison WR, Karkalas J (1990) Starch. In: Day PM (ed) Methods in Plant Biochemistry, Academic Press, San Diego, California USA

Mouille G, Maddelein M-L, Libessart N, Talaga P, Decq A, Delrue B, Ball S (1996) Preamylopectin processing: A mandatory step for starch biosynthesis in plants. Plant Cell 8:1353-1366

Mukerjea R, Robyt JF (2004) Starch biosynthesis: the primer nonreducing-end mechanism versus nonprimer reducing-end two-site insertion mechanism. Carbohydrate Res 340:245-255

Muller-Rober B, Sonnewald U, Willmitzer L (1992) Inhibition of the ADP-glucose pyrophosphorylase in transgenic potatoes leads to sugar-storing tubers and influences tuber formation and expression tuber storage protein genes. EMBO J 11:1229-1238

Munoz FJ, Baroja-Fernandez E, Moran-Zorzano MT, Viale AM, Etxeberria E, Alonso-Casajus N, Pozueta-Romero J (2005) Sucrose synthase controls both intracellular ADP glucose levels and transitory starch biosynthesis in source leaves. Plant Cell Physiol 46:1366-1377

Myers AM, Morell MK, James MJ, Ball SG (2000) Recent progress toward understanding biosynthesis of the amylopectin crystal. Plant Physiol 122:989-997

Nachtergaele W, Van Nuffel J (1989) Starch as a stilt material in carbonless copy paper-new developments. Starch/Starke 41:386-392

Nakamura T, Yamamori M, Hirano H, Hidaka S, Nagamine T (1995) Production of waxy (amylose-free) wheats. Molec General Genet 248:253-259

Nakamura Y, Kubo A, Shimamune T, Matsuda T, Harada K, Satoh H (1997) Correlation between activities of starch debranching enzyme and α-polyglucan structure in endosperms of *sugary-1* mutants of rice. Plant J 12:143-153

Nakano A, Suzuki G, Yamamoto M, Turnbull K, Rahman S, Mukai Y (2005) Rearrangements of large-insert T-DNAs in transgenic rice. Mol Gen Genomics 273:123-129

Nielsen TM, Deiting U, Stitt M (1997) A beta-amylase in potato tubers is induced by storage at low temperature. Plant Physiol 113:503-510

Nishi A, Nakamura Y, Tanaka N, Satoh H (2001) Biochemical and genetic analysis of the effects of *amylose extender* mutation in rice endosperm. Plant Physiol 127:459-472

Oleykowski CA, Mullins CRB, Godwin AK, Yeung AT (1998) Mutation detection using a novel plant endonuclease. Nucl Acids Res 26:4597-4602

Patron NJ, Greber B, Fahy BF, Laurie DA, Parker ML, Denyer K (2004) The *lys5* mutations of barley reveal the nature and importance of plastidial ADP-Glc transporters for starch synthesis in cereal endosperm. Plant Physiol 135:2088-2097

Peng MS, Hucl P, Chibbar RN (2001) Isolation, characterization and expression analysis of starch synthase I from wheat (*Triticum aestivum* L.). Plant Sci 6:1055-1062

Peng MS, Gao M, Båga M, Hucl P, Chibbar RN (2000) Starch-branching enzymes preferentially associated with A-type starch granules in wheat endosperm. Plant Physiol 124:265-272

Perry JA, Wang TL, Welham TJ, Gardner S, Pike JM, Yoshida S, Parniske M (2003) A TILLING reverse genetics tool and a web-accessible collection of mutants of the legume *Lotus japonicus*. Plant Physiol 131:866-871

Preiss J, Sivak MN (1998) Starch synthesis in sinks and sources. In: Zamaski E, Schaffer AA (eds) Photoassimilate distribution in plants and crops-Source-sink relationships. Marcel Dekker Inc. New York, Basel, Hong Kong, pp 63-96

Raemakers CJJM, Schreuder MM, Pereira I, Suurs L, Vincken JP, Jacobsen E, Visser RGF (2003) Production of amylose-free cassava plants by genetic modification. In**:** Fauquet CM, Taylor NJ (eds) Cassava: An ancient crop for modern times [Compact Disc 2]. Proceedings of the 5th International Meeting of the Cassava Biotechnology Network (4-9 November 2001, St Louis, MO)

Rashid H, Yokoi S, Toriyama K, Hinata K (1996) Transgenic plant production mediated by *Agrobacterium* in indica rice. Plant Cell Rep 15:727-730

Regina A, Bird A, Topping D, Bowden S, Freeman J, Barsby T, Kosar-Hashemi B, Li ZY, Rahman S, Morell M (2006) High-amylose wheat generated by RNA interference improves indices of large-bowel health in rats. Proc Natl Acad Sci USA 103:3546-3551

Sakulsingharoj C, Choi SB, Hwang SK, Edwards GE, Bork J, Meyer CR, Preiss J, Okita TW (2004) Engineering starch biosynthesis for increasing rice seed weight: the role of the cytoplasmic ADP-glucose pyrophosphorylase. Plant Sci 167:1323-1333

Shannon JC, Pien F-M, Cao H, Liu K-C (1998) Brittle-1, an Adenylate translocator, facilitates transfer of extraplastidial synthesized ADP-glucose into amyloplasts of maize endosperm. Plant Physiol 117:1235-1252

Seib P (2000) Reduced-amylose wheats and Asian noodles. Cereal Foods World 45:504-512

ShiF, Hoekstra D (2004) Effective intracellular delivery of oligonucleotides in order to make sense of antisense. J Con Rel 97:189-209
Singletary GW, Banisadr R, Keeling PL (1994) Heat stress during grain filling in maize: Effects on carbohydrate storage and metabolism. Aust J Plant Physiol 21:829-841
Slade AJ, Fuerstenberg SI, Loeffler D, Steine MN, Facciotti D (2005) A reverse genetic, nontransgenic approach to wheat crop improvement by TILLING. Nat Biotech 23:75-81
Slattery CJ, Kavakli H, Okita TW (2000) Engineering starch for increased quantity and quality. Trends Plant Sci 5:291-298
Smidansky ED, Clancy M, Meyer FD, Lanning SP, Blake NK, Talbert LE, Giroux MJ (2002) Enhanced ADP-glucose pyrophosphorylase activity in wheat endosperm increases seed yield. Proc Natl Acad Sci USA 99:1724-1729
Smith AM (2001) The biosynthesis of starch granules. Biomacromolecules 2:335-341
Smith AM, Denyer K, Martin C (1997) The synthesis of the starch granule. Annu Rev Plant Physiol Plant Mol Biol 48:67-87
Song J, Bradeen JM, Naess SK , Helgeson JP (2003) BIBAC and TAC clones containing potato genomic DNA fragments larger than 100 kb are not stable in *Agrobacterium*. Theor Appl Genet 107:958-964
Stark DM, Timmerman KP, Barry GF, Preiss J, Kishore GM (1992) Regulation of the amount of starch in plant-tssues by ADP- Glucose Pyrophosphorylase. Science 258:287-292
Stroh WH (1997)Starch: The essential food and ingredient. Spectrum Food Industry Decision Resources Inc. 79:1-16
Takeda Y, Preiss J (1993) Structures of B90 (*sugary*) and W64A (normal) maize starches. Carbohydr Res 240: 265-275
Takeda Y, Hizukuri S (1987) Structures of branched molecules of amylose of various origins and molar fractions of branched and unbranched fractions. Carbohydr Res 165:139-145
Takeda Y, Hizukuri S, Juliano BO (1986) Purification and structure of amylose from rice starch. Carbohydr Res 148:299-308
Tester RF, Karkalas J (2002) Polysaccharides. II. Polysaccharides from eukaryotes. In: Vandamme EJ, De Bates S, Steinbuchel A (eds) Starch in biopolymers. vol 6, Weinheim, Wiley-VCH, pp 381-438
Thorbjørnsen T, Villand P, Denyer K, Olsen O-A, Smith AM (1996) Distinct isoforms of ADP glucose pyrophosphorylase occur inside and outside the amyloplasts in barley endosperm. Plant J 10:243-250
Till BJ, Reynolds SH, Greene EA, Codomo CA, Enns LC, Johnson JE, Burtner C, Odden AR, Young K, Taylor NE, Henikoff JG, Comai L, Henikoff S (2003) Large-scale discovery of induced point mutations with high-throughput TILLING. Genome Res 13:524-530
Till BJ, Reynolds SH, Wei C, Springer N, Burtner C, Young K, Bowers E, Codomo CA, Enns LC, Odden AR, Greene EA, Comai L, Henikoff S (2004) Discovery of induced point mutations in maize genes by TILLING. BMC Plant Biol 4:12

Tillet IJL, Bryce JH (1993) The regulation of starch granule size in endosperm of developing barley grains. In: Proc 24th Eur Brewert Convention Congress, Oslo, Norway, pp 45-52

Tsutsumi N, Kanayama K, Tano S (1992) Suppression of α-amylase gene expression by antisense oligodeoxynucleotide in barley cultured aleurone layers. Jpn J Genet 67:147-154

Visser RG, Somhorst I, Kuipers GJ, Ruys NJ, Feenstra WJ, Jacobsen E (1991) Inhibition of the expression of the gene for granule-bound starch synthase in potato by antisense constructs. Mol Gen Genet 225:289-296

Waterhouse PM, Graham MW, Wang M-B (1998) Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. Proc Natl Acad Sci USA 95:13959-13964

Waterhouse PM, Helliwell CA (2002) Exploring plant genomes by RNA-induced gene silencing. Nat Genet Rev 4:29-38

Yamguchi I, Otobe C, Uchiyama T, Yanagisawa T, Seko H, Amano Y, Yoshida H (2000) Wheat starch with novel characteristics. US patent 6,165,535

Yamamori M, Fujita S, Hayakawa K, Matsuki J, Yasui T (2000) Genetic elimination of a starch granule protein, SGP-1, of wheat generates an altered starch with apparent high amylose. Theor Appl Genet 101:21-29

Yasui T, Matsuki J, Sasaki T, Yamamori M (1996) Amylose and lipid contents, amylopectin structure, and gelatinisation properties of waxy wheat (*Triticum aestivum*) starch. J Cer Sci 24:131-137

Young TE, Juvik JA, DeMason DA (1997) Changes in carbohydrate composition and a-amylase expression during germination and seedling growth of starch-deficient endosperm mutants of maize. Plant Sci 129:175-189

Zeeman SC, Umemoto, Lue W-L, Au-Yeung P, Martin C, Smith AM, Chen J (1998) A mutant of *Arabidopsis* lacking a chloroplastic isoamylase accumulates both starch and phytoglycogen. Plant Cell 10:1699-1711

Zhang X, Colleoni C, Ratushna V, Sirghie-Colleoni M, James MG, Myers AM (2004) Molecular characterization demonstrates that the *Zea mays* gene *sugary2* codes for the starch synthase isoform SSIIa. Plant Mol Biol 54:865-879

8

Bioethanol: role and production technologies

David Chiaramonti

Research Center for Renewable Energies, Department of Energetics "Sergio Stecco", Faculty of Mechanical Engineering, University of Florence, Via di S. Marta 3, 50139 Florence, Italy (e-mail: david.chiaramonti@unifi.it)

1. Introduction

Bioethanol (CH_3CH_2OH) is a liquid biofuel which can be produced from several different biomass feedstocks and conversion technologies. Its main physical and chemical characteristics, compared to diesel and gasoline fuels, are given in the Table 8.1.

Table 8.1. Main physical and chemical properties of bioethanol (Source: DG XII 1994; Blondy 2005; Mc Cormick et al. 2001).

	Diesel	**Ethanol**	**Gasoline**
Low Heating Value - LHV (MJ/kg)	42.7	26.9	43.7
Low Heating Value - LHV (MJ/l)	36.4	21.0	32.0
Viscosity (cSt)	2.5	-	-
Density (kg/m^3) @ 15°C	830 - 880	790	700 - 780
Cetane number	> 45	below 8	-
Octane number (MON)	-	96 - 106	79 - 98
Stoichiometric ratio	14.5	9	15.1
Vapour pressure @ 38°C (psi)	0.04	2.5	7-9
Flash point (°C)	55-65	13	-40
Boiling temperature (°C)	170-340	78	33-213
Vaporization heat (kJ/kg)	-	842	300
Auto-ignition temperature (°C)	230-315	366	300-371
Flammability limits (°C)	64-150	13-42	(-40)-(-18)
Flammability limits (% vol)	0.6-5.6	3.3-19.0	1.4-7.6

P. Ranalli (ed.), Improvement of Crop Plants for Industrial End Uses, 209–251.

Bioethanol can be used as chemical in industrial applications, as fuel for energy generation, or as food. It can be produced by synthesis or by fermentation processes, and be or not denatured. Ethanol can be used neat or blended with gasoline or diesel fuels. It is also used for the production of ETBE (Ethyl Tertiary Butyl Ether), an oxygenated fuel obtained by mixing ethanol and isobutylene and reacting them with heat over a catalyst. ETBE promotes clean gasoline combustion, thus improving air quality.

2. Bioethanol market

Biomass currently supply 4 % of EU energy needs from biomass (Biomass Action Plan, 2005). The increased use of biomass is expected to generate a wide range of benefits and advantages, such as a greater diversification of energy supply and reduction of energy imports, reduction of greenhouse gas emissions, and creation of new permanent jobs, especially in the rural areas.

Bioethanol is playing a very important role in the liquid fuel market: bioethanol worldwide production was greater than 18 Mt/y in 2003, sig-

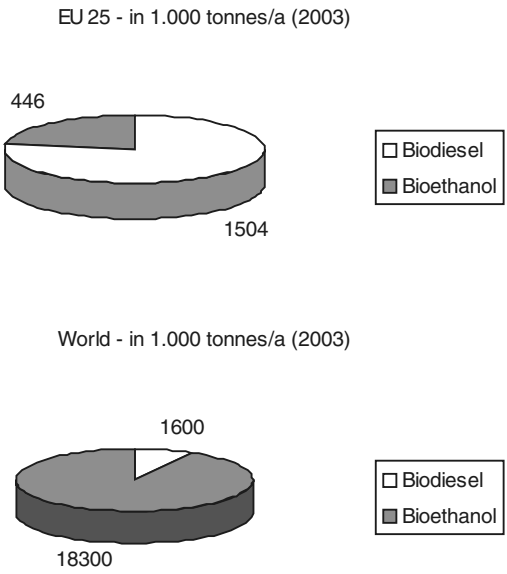


Fig. 8.1. EU-25 and World bioethanol production (from Biofuel Barometer, June 2004).

nificantly higher than biodiesel (approximately 1.6 Mt/y worldwide production). The characteristics of the biofuel sector however are very different in USA or EU-25, where biodiesel production was more than three times higher than bioethanol. In energy terms, at world level bioethanol represented more then 89 % of the energy in biofuels in 2003 (considering both bioethanol and biodiesel), while it was below 18 % in EU-25.

Bioethanol accounted for 2.8 % of motor gasoline worldwide, while biodiesel reached only 0.2 % (1 % in EU).

The most important bioethanol production Countries in the world are Brazil and US/Canada, as shown in Figure 8.2.

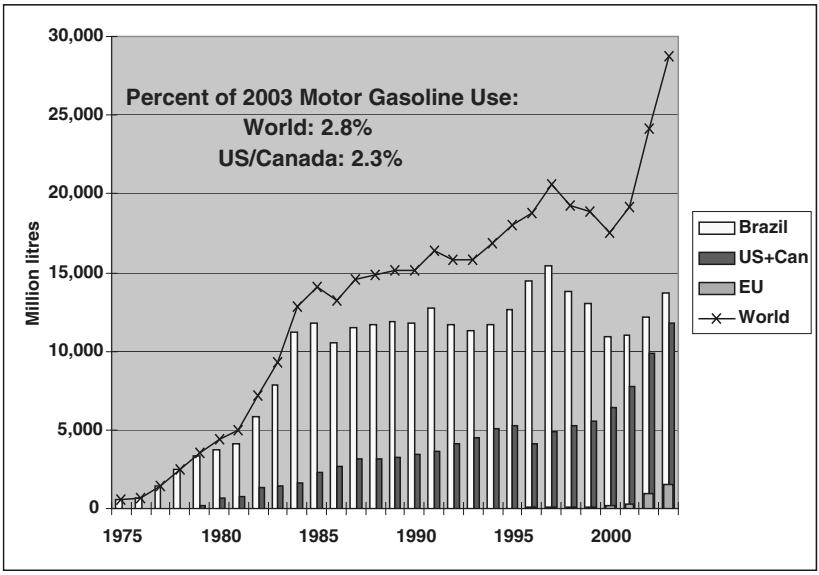


Fig. 8.2. World bioethanol production (Source: Fulton L. 2004; F.O. Licht).

Sugar cane is the main feedstock for bioethanol production in Brazil, while corn and sugar beet are the major resources in US and EU respectively.

The increased biofuel production in the EU (EU-25 considered only for 2004) is illustrated in Figure 8.3.

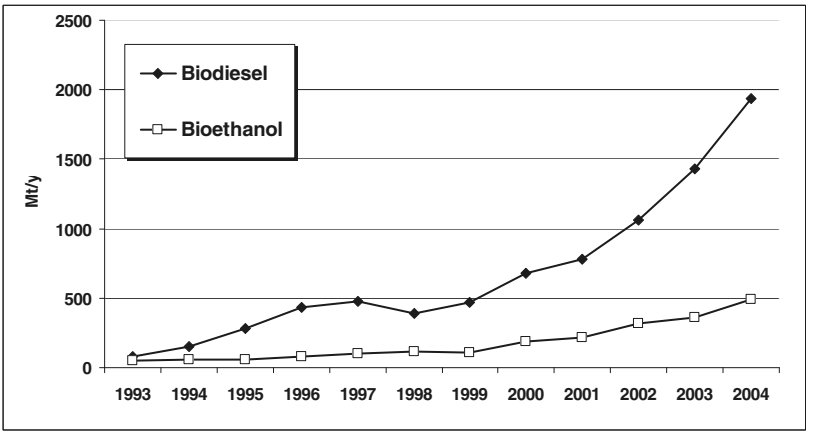


Fig 8.3. Bioethanol production in EU (from Eurobserv'ER, www.energies-renouvelables.org).

The distribution of bioethanol and ETBE production among the various EU Countries is reported in the next graph ("EC" means wine alcohol transformed into bioethanol as automotive fuel and marketed by the European Commission, in the framework of the common wine market management).

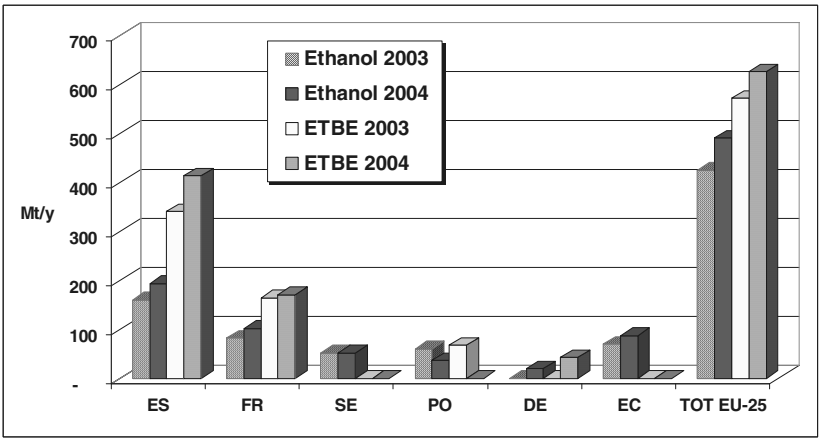


Fig. 8.4. Bioethanol and ETBE production (from Eurobserv'ER, www.energies-renouvelables.org).

In 2003, three EU Countries, ES, FR and PO, produced 96 % of EU bioethanol/ETBE: 5 EU Member States only were producing bioethanol/ETBE at that time.

In 2003, community legislation set ambitious targets for biofuels (Directive 2003/30/EC of 8 May 2003 on the promotion of the use of biofuels or other renewable fuels for transports, OJ L123 of 17 May 2003, page 42). This "Biofuel" Directive states that Member States must ensure by end of 2005 a 2% and by end 2010 a 5.75% minimum proportion of biofuels of

Table 8.2. Biofuel progress at National level (EU-25) towards Biofuel Directive (Source: Biomass Action Plan 2005).

Member State	Market share 2003	National indicative target for 2005	Targeted increase 2003-2005
AT	0.06%	2.5%	+2.44%
BE	0%	2%	+2%
CY	0%	1%	+1%
CZ	1.12%	3.7% (2006)	+ 1.72% (assuming linear path)
DK	0%	0%	+0%
EE	0%	2%	+2%
FI	0.1%	0.1%	+0%
FR	0.68%	2%	+1.32%
DE	1.18%	2%	+0.82%
GR	0%	0.7%	+0.7%
HU	0%	0.4-0.6%	+0.4-0.6%
IE	0%	0.06%	+0.06%
IT	0.5%	1%	+0,5%
LA	0.21%	2%	+1.79%
LI	0% (assumed)	2%	+2%
LU	0% (assumed)	not yet reported, assume 0%	not yet reported
MT	0.02%	0.3%	+0.28%
NL	0.03%	2% (2006)	0% (promotional measures will come into force from January 2006)
PL	0.49%	0.5%	+0.01%
PT	0%	2%	+2%
SK	0.14%	2%	+1.86%
SI	0% (assumed)	0.65%	+0.65%
ES	0.76%	2%	+1.24%
SV	1.32%	3%	+1.68%
UK	0.03%	0.3%	+0.27%
EU25	0.6%	1.4%	+0.8%

all gasoline and diesel fuels sold on their market. Biofuels can be made available as pure (neat) fuels, blended in mineral oil derivatives or liquids derived from biofuels such as ETBE.

A recent communication and assessment from the European Commission (Biomass Action Plan, 2005) concluded that the 2005 reference value will not be achieved: assuming that all Member States are able to meet the targets they have set, only a share of 1.4 % will be reached.

About 90 % of biofuel consumption is covered by domestic raw material, 10 % by imports. Approximately 1.8 million hectares (out of 97 million hectares in EU-25) were used for biofuel production in 2005. The observed trend shifted towards low blends and away from high blends or pure fuels, that were the main part in 2001. A report on Directives' implementation will be elaborated by the Commission in 2006.

However, various authors are expecting a fast growth of bioethanol in the EU, thanks to improved lignocellulosic-to-ethanol processes, currently at demonstration stage.

More recent market estimations for bioethanol in 2010 give the figures reported in the following table**.**

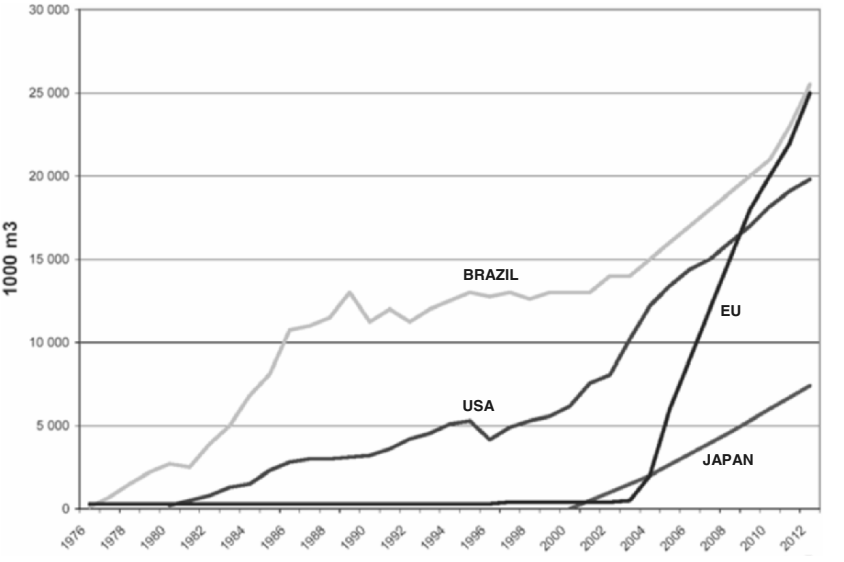


Fig 8.5. Future trends in bioethanol markets (source: Luiz Otavio Laydner, CFA Banco Pactual, Brazil).

Table 8.3. Bioethanol market estimations at 2010 (Piacente and Wolter 2005).

Country/region	Billion liters
Brazil	21
USA	18-20
Europe (EU)	9-14
Japan	6
South Korea	1.9
China	4.8
Eastern Europe	1-2
Canada	1-2
Total	**60-75**

As regards supporting measures to biofuels in the EU, two main instruments are adopted by Member States for promoting the use of liquid biofuels for transports:

- Tax exemption
- Biofuel obligation

Tax exemption can be implemented under the Article 16 of the Directive 2003/96/EC of 27/10/2003 restructuring the Community framework for the taxation of energy products and electricity (OJ L 283 of 31.10.2003 page 51), which allows Member States to apply exemption or reduced rate of taxation to biofuels and other products produced from biomass. Several EU Countries, such as Germany, France, Italy, Sweden, United Kingdom, received state aid approval to biofuel tax exemption or reduction measures. This approach, however, requires that tax reductions or exemptions are modified in line with changes in the price of raw materials, in order to avoid overcompensation. This represents a first risk of such measure: other possible problems due to adoption of tax exemption measures are related to investor uncertainty (the Energy Taxation Directive limits the duration of tax exemptions to 6 years only), and the adoption of quota-based approaches, that create risks of non-transparency, arbitrary allocation, and increased market concentration (Biomass Action Plan 2005).

Biofuels obligations instead require that fuel supply companies incorporate a given percentage of biofuels in the fuel they place on the national market. It is a simpler and effective way to promote the introduction of higher amounts of biofuels, as the problem of oil dependence is addressed to the sector where it is originated. Moreover, these measures are not subject to time limits and establish sound and stable frameworks, which are basic requirements for investors.

A further technical barrier to biofuels is represented by the Directive 98/70/EC of 13 October 1998 relating to the quality of petrol and diesel fuels (OJ L 350, 28.12.1998), as amended by Directive 2003/17/EC of 3 March 2003 (OJ L 76, 22.3.2003). By limiting the vapour pressure of petrol, limits are established on ethanol, ether and other oxygenates content. The Commission is reviewing the fuel quality directive.

3. Production technologies

Bioethanol can be produced from (i) sugar or starch crops (as sugar cane, sugar beet, corn and wheat), and from (ii) lignocellulosic biomass. While production technologies are well known and developed for microbiological fermentation of sugar and starch crops, bioethanol from lignocellulosic via acid/enzymatic hydrolysis or thermochemical processes is not yet fully developed at industrial scale (Hamelink et al. 2005).

Table 8.4. Typical bioethanol yields from different feedstocks (Schieder 2005).

Crop	Expected bioethanol yield (lt/ha/y)
Grain	1,800 – 2,500
Corn	up to 3,800
Sugar cane	up to 5,600
Sugar beet	up to 7,000
Lignocellulosic biomass	up to 9,000

The production of bioethanol from lignocellulosic biomass represents a very promising alternative able to significantly increase feedstock availability and to reduce feedstock costs, but requires the industrialization of innovative processes and technologies currently at pilot/demonstration stage. Various conversion processes can be employed to produce bioethanol from lignocellulosic biomass.

Bioethanol from sugar/starch crops through traditional production technologies is included in the group of "1st Generation Biofuels" (together with Pure Plant Oil and Biodiesel obtained from Vegetable Oil estherification), while bioethanol from lignocellulosic biomass is considered as a "2nd Generation Biofuel".

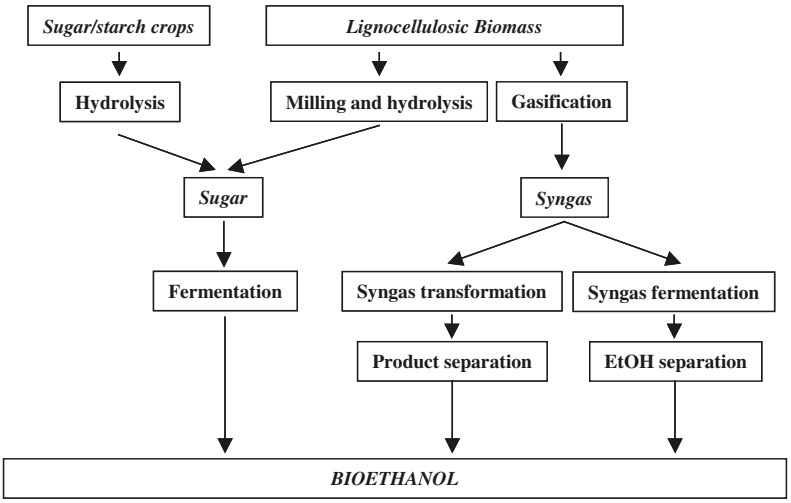


Fig. 8.6. Main pathways for bioethanol production (modified from Hamelinck et al. 2005; Spath and Dayton 2003).

3.1 Bioethanol from sugar or starch crops

Bioethanol production processes from sugar or starch crops are the most traditional and developed pathways. Fermentation is performed by microorganisms (yeasts, bacteria, fungi) in the absence of oxygen according to the following main reactions:

$$C_6H_{12}O_6 \rightarrow 2\,C_2H_5OH + 2\,CO_2$$

The theoretical maximum yield of ethanol is 0.5111 kg of bioethanol and 0.4889 kg of CO_2 per kg of sugar. *Saccharomices cerevisiae* is the typical yeast for hexose sugars fermentation.

The fermentation of 5-carbon sugars (pentoses, such as arabinose and xylose) derived from lignocellulosic biomass is also possible (see next chapters), but not with ordinary strains of yeasts, such as *Saccharomices c.*

$$3\,C_5H_{10}O_5 \rightarrow 5\,C_2H_5OH + 5\,CO_2$$

3.1.1 Bioethanol from sugar cane and sugar beet

Sugar Cane. Sugar cane is initially milled to extract sugars, which amount corresponds to 12-17 % (90% saccharose and 10 % glucose): the extraction efficiency is around 95 %. The remaining solid product is the bagasse which consists of lignocellulosic material.

After extraction, which is different according to the type of distillery (producing ethanol only, or ethanol and sugar), the sugar content has to be adjusted to 14-18% to achieve optimum fermentation efficiency of yeast, the most common being *Saccharomyces c.*, at temperatures around 33-35 °C and cell density of 8-17 % v/v (Zimbardi et al. 2002). Fermentation is interrupted at ethanol concentration of approximately 10 %: then, the broth is sent to the distillation and rectification phase, which product is an azeotropic solution of 95 % v/v ethanol. Further concentration to absolute ethanol (high grade or anhydrous ethanol) is finally achieved by molecular sieves or distillation using benzene or cyclohexane (azeotropic distillation).

The co-product of the distillation phase is the "vinasse", which amount is 10-15 time the ethanol production, and that is sent to the cane fields for fertilisation and irrigation purposes.

The solid lignocellulosic residue, the bagasse, can be used for cogeneration (simultaneous generation of electricity and heat), so to provide the process with the necessary energy input. This also results in a further reduction of greenhouse gas emission, as the bagasse is biomass, i.e. a renewable fuel.

Sugar Beet. Sugar beet is also a typical feedstock for bioethanol production. Differently from sugar cane, which is first crushed and squeezed to mechanically extract the juice, the sugar beet slices are initially treated by contact with water or beet juice at 70-80°C. Temperature is a critical parameter for the diffusion process as it has to break down the proteins in the cell walls. The juice is fermented by yeast (as *Saccharomyces c.*) or bacteria: the use of bacteria is however still at batch level. The pulp, once drained, is used as animal feed or sold to the chemical or pharmaceutical industry (substrate for the production of citric acid and its esters).

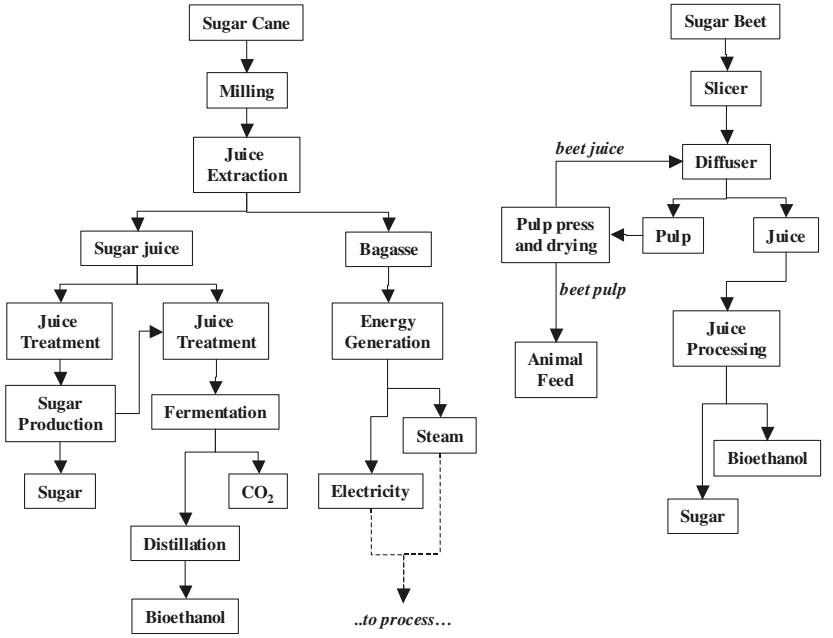


Fig. 8.7. Main processes for bioethanol production from sugar cane and sugar beet (Zimbardi et al. 2002).

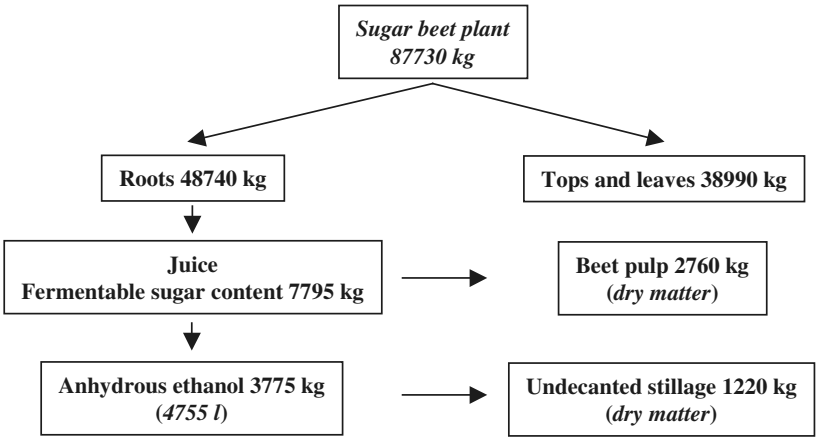


Fig. 8.8. Mass balance of sugar beet to ethanol (source: DGXII 1994).

3.1.2 Bioethanol from starch crops

Starch crops (as corn, wheat, barley, oat, etc) are also other typical feedstocks for bioethanol production through traditional technologies. Starch is a polymer constituted by glucose molecules (monomers) C-O bonded together in different stereoisomerisms (α or β bonds).

The most used milling processes in starch-to-bioethanol production plants are (i) wet milling or (ii) dry milling (Zimbardi et al. 2002).

- In the wet process (DGXII 1994), after backing and saccharification, a glucose solution is obtained (starch process)
- In the dry process, a rude home grinding of the grains is carried out, followed by a backing-liquefaction in hot water.

Wet milling. The traditional wet milling process starts with softening the grains (steeping) in a 0.1-0.2 % SO_2 water solution at 50°C for 1-2 days. The softened grains are then milled: oil, protein and starch-rich are separated, while the concentrated (50 % v/v) steep water becomes Corn Steep Liquor and the remaining liquid is used for adjusting the pH (4-4.5) of the saccharification process, supply nutrients to the fermentation process, and make the fermentation broth sufficiently diluted to make it compatible with the yeasts.

The gelatinised starch fraction, after addition of NaOH at 70°C (so that pH becomes 5.5-6.2), is liquefied (DGXII, 1994) by adding the α-amylase enzyme (stabilised by adding $CaCl_2$). Dextrins are produced during this phase. Maltose is a disaccharide composed by linking two glucose molecules (monosaccharides): it is the typical product of starch digestion. Other disaccharides are sucrose (glucose + fructose: it is the common table sugar) and lactose (glucose + galactose: the main sugar in milk). Glucose, galactose and fructose are 3 structural isomer sugars.

Starch hydrolysis (liquefaction)

$$2\,n\,C_6H_{12}O_{11} + n\,H_2O \xrightarrow{(\alpha\text{-amylase})} n\,C_{12}H_{22}O_{11}$$
starch *water* *maltose*

Dextrins are then converted into glucose by the glucoamylase enzyme (saccharification).

$$C_{12}H_{22}O_{11} + H_2O \xrightarrow{(glucoamylase)} 2\,C_6H_{12}O_6$$
maltose *water* *glucose*

$$C_{12}H_{22}O_{11} + H_2O \xrightarrow{(invertase)} C_6H_{12}O_6 + C_6H_{12}O_6$$
saccharose *water* *glucose* *fructose*

Finally, fermentation (i.e. conversion of monosaccharides into ethanol and CO_2) is carried out by yeast (again, the most used is *Saccharomyces c.*) at lower temperature (~29-35°C). Bioethanol and CO_2 can then be recovered for various applications (for the food industry, for dry ice making, etc).

$C_6H_{12}O_6$	→	$2\ C_2H_5OH$	+	$2\ CO_2$
glucose, fructose		*bioethanol*		*carbon dioxide*

450 g of yeasts must be added per 1000 l of mash, corresponding to about 5-10 mill.cells per ml (DG XII, 1994). The sugar concentration should be 16-24 % to achieve a bioethanol production 8-12 %, with a pH of 4.5 – 5. Theoretical yields are 0.568 kg EtOH / kg starch, and 0.538 kg EtOH / kg saccharose.

Fermentation can be performed as a batch or a continuous process, depending on the plant. Above 8-10 % v/v concentration yeasts cannot survive anymore: the liquor is then distilled to 95 % v/v concentration. Distillation is performed in column: it is a very energy-demanding part of the entire process. Recently (Grassi et al. 2005), low-cost crystal hydrated compounds have been proposed to achieve a significant reduction of energy demand for bioethanol separation.

Depending on plant type, before distillation yeasts can be recovered by centrifugation.

Anhydrous ethanol (absolute ethanol, 99 % v/v) can finally be obtained through dehydratation by molecular sieves or other similar processes.

In case gluten is not recovered (Modified Wet Milling process), the "stillage" which remains after fermentation (10-15 times the ethanol volume) is used to produced Distillers' Dried Grain (DDG) and Distillers' Dried Soluble (DDS), that have a market as animal feed thanks to their nutritional value (proteins, fibre, fats).

Dry milling. Grains are here directly milled, without pre-treatment: after water addition and heating, the slurry is then hydrolysed, fermented and distilled as in the wet milling process. While wet milling assure the production of purer starch and higher value co-products, dry milling offer some advantages such as lower power demand, lower investment costs, and higher bioethanol production.

A summary of the processes described above is reported in the next tables for the corn-to-ethanol process.

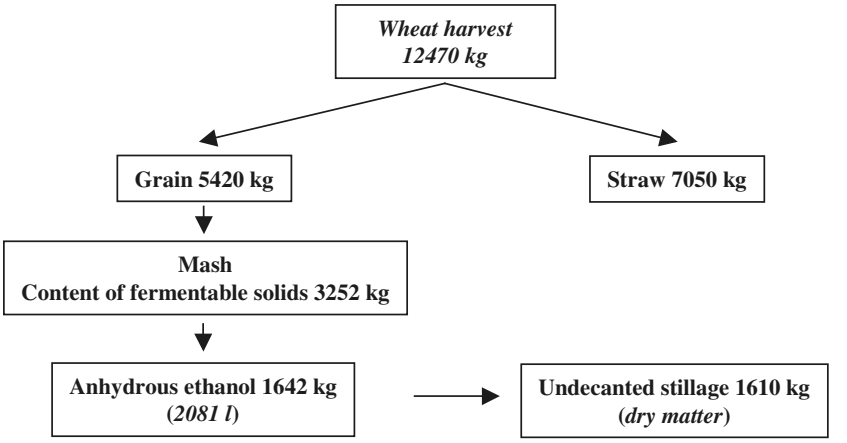


Fig. 8.9. Mass balance of wheat to ethanol (source: DGXII 1994).

Table 8.5. Main products from wet and dry milling corn-to-ethanol processes.

	Wet Milling Processes	Dry Milling Process	Applications
Bioethanol	Yes	Yes	Fuel, chemical
CO_2	Yes, but not always recovered	Yes, but not always recovered	Food industry, dry ice
Corn Oil	Yes	No	Human consumption
Gluten Feed	Yes	No	Animal feed (protein content: ~20 % wt dry basis)
Gluten Meal	Yes	No	Animal feed (protein content: ~60 % wt dry basis)
Corn Steep Liquor (CSL)	Yes	No	Animal feed (protein content: ~50 % wt dry basis)
DDG-DDS (DDGS)	No	Yes	Animal feed

3.2 Bioethanol from lignocellulosic biomass

Lignocellulosic biomass today represents the most interesting and promising feedstock for bioethanol production. It is abundant and widely available (either from woody or herbaceous crops, various kind of wastes, etc).

The main constituents of lignocellulosic biomass are cellulose, hemicellulose, lignin, extractives and ash (Van Loo and Koppejan 2002; Bryden 1998, Zimbardi et al. 2002).

- Cellulose $(C_6H_{10}O_5)_n$ is a high molecular weight linear polymer, consisting of chains of bonded glucose monomers ($C_6H_{12}O_6$). Fibers walls mainly consist of cellulose, which accounts for 30-60 % of wood (lignocellulosic) dry weight. It is a rigid, ordered compact polymer, and resistant to chemical attack.
- Hemicellulose is a short branched polymer similar to cellulose, but built from several sugars that encase cellulose fibres. It consists of short, highly branched, chains of sugars. It contains five-carbon sugars (usually D-xylose and L-arabinose) and six-carbon sugars (D-galactose, D-glucose and D-mannose) and uronic acid. It corresponds to 10-40 % of wood dry weight. Part of hemicellulose is composed by 5-carbon sugar (as xylose and arabinose), the remaining by 6-carbon sugars (glucose, mannose, galactose). The amount of 6-carbon sugars is higher in softwood, while hardwood contains more 5-carbon (xylose) sugars. Hemicellulose is amorphous and relatively easy to hydrolyze to its constituent sugars.
- Lignin is a three-dimensional non-sugar polymer which strength the wood fibers. It accounts for 10-30 % of wood (lignocellulosic) dry weight.
- Extractives are non structural components (terpenes, fats and waxes, phenolics) that give the biomass a wide range of properties as taste, odor, color, etc. They can be "extracted" by using solvents such as water, benzene or alcohol. Their amount is up to 5-10 % dry-weight.
- Ashes are inorganic salts contained in biomass.

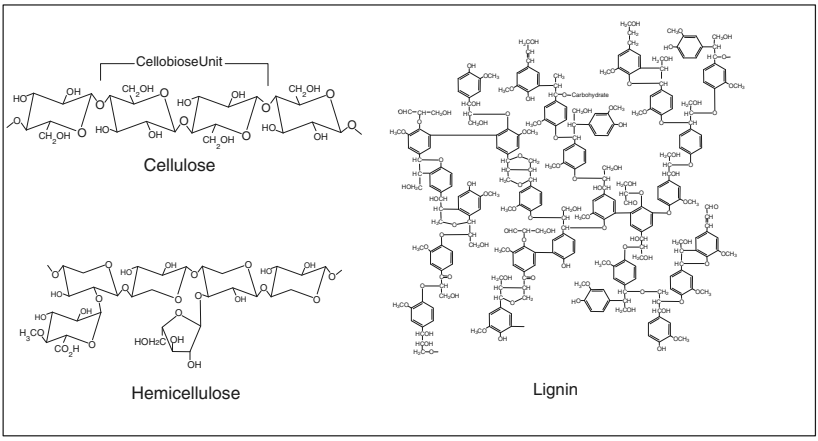


Fig. 8.10. Cellulose, hemicellulose and hypothesized lignin chemical structure (reprinted from Zimbardi et al. 2002).

Table 8.5. Composition of various types of biomasses (source: Sun and Cheng 2002).

Lignocellulosic materials	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwoods stems	40–55	24–40	18–25
Softwood stems	45–50	25–35	25–35
Nut shells	25–30	25–30	30–40
Corn cobs	45	35	15
Grasses	25–40	35–50	10–30
Paper	85–99	0	0–15
Wheat straw	30	50	15
Sorted refuse	60	20	20
Leaves	15–20	80–85	0
Cotton seed hairs	80–95	5–20	0
Newspaper	40–55	25–40	18–30
Waste papers from chemical pulps	60–70	10–0	5–10
Primary wastewater solids	8–15	Not available	24–29
Coastal Bermuda grass	25	35.7	6.4
Switch grass	45	31.4	12.0

Table 8.6. Typical lignocellulosic biomass composition (source: Hamelinck et al. 2005).

Feedstock	Hard wood			Softwood	Grass
	Black locust	Hybrid Poplar	Eucalyptus	Pine	Switchgrass
Cellulose	41.61	44.70	49.50	44.55	31.98
Glucan 6C	*41.61*	*44.70*	*49.50*	*44.55*	*31.98*
Hemicellulose	17.66	18.55	13.07	21.90	25.19
Xylan 5C	*13.86*	*14.56*	*10.73*	*6.30*	*21.09*
Arabinan 5C	*0.94*	*0.82*	*0.31*	*1.60*	*2.84*
Galactan 6C	*0.93*	*0.97*	*0.76*	*2.56*	*0.95*
Mannan 6C	*1.92*	*2.20*	*1.27*	*11.43*	*0.30*
Lignin	26.70	26.44	27.71	27.67	18.13
Ash	2.15	1.71	1.26	0.32	5.95
Acids	4.57	1.48	4.19	2.67	1.21
Extractives	7.31	7.12	4.27	2.88	17.54
Heating value (GJ_{HHV}/t_{DM})	19.5	19.6	19.5	19.6	18.6

Various demonstration projects are currently ongoing on ethanol production from lignocellulosic biomass (Biomass Action Plan, 2005). A first 4 million liters plant was realised and operated by Iogen in Ottawa (Canada), followed by a 150,000 liters facility in Sweden by ETEK. A third 5 million liter unit is under construction in Spain by Abengoa (Zimbardi *et al*. 2002).

3.2.1 Hydrolysis

The hydrolysis process consists of breaking the carbohydrates that constitute the biomass into monomeric sugars, which can then be fermented by microorganisms to produce bioethanol. Two main hydrolysis methods are used:

- Chemical hydrolysis: it can be carried out as (i) Concentrated Acid Hydrolysis or (ii) Dilute Acid Hydrolysis
- Enzimatic hydrolysis

Concentrated Acid Hydrolysis. The process (Zimbardi et al. 2002) starts with hemicellulose hydrolysis in a heated 10 % sulphuric acid solution (two hours) and lignin removal. 5-carbon and 6-carbon solubilised sugars are then separated from the solid residue, which mainly consists of cellulose and lignin, and sent, after neutralization, to the fermentation unit.

After hemicellulose hydrolysis and lignin recovery, a second stage of cellulose hydrolysis is therefore performed: cellulose is converted to sugars according to the following reaction (Hamelinck et al. 2005): $(C_6H_{10}O_5)n + nH_2O \rightarrow nC_6H_{12}O_6$.

The solid residue is mixed with a 30 % acid solution, which is then further concentrated up to 70 % by heating. The crystalline structure of the cellulose is here broken. Suspension in hot water into a hydrolytic tank is afterwards implemented, in which 10 % acid concentration media is realised, and heating at 100°C for two hours is carried out.

The solution, which contains 10 % acid and 10 % glucose after the cellulose has been completely hydrolysed, is neutralised by adding Calcium hydroxide $Ca(OH)_2$: the hydrated gypsum ($CaSO_4\ 2H_2O$) which is formed precipitates and is removed, and sugars are finally conveyed to the fermentation plant. The use of the filtered gypsum is an important aspect of the process, especially as regards its economical performances: it could be used in agriculture (as a soil conditioner) or represent a waste to be disposed of. Gypsum production range from 0.02 kg/kg feedstock to 0.6-0.9 kg/kg feedstock, depending on the acid recycling strategy.

The efficiency of this process ranges from 90-96 % for hemicellulose to xylose (5-carbon sugar) to 79-90 % for cellulose to glucose (6-carbon), depending on feedstock type. The process therefore guarantees very high total yields, in the order of 90 %.

Variations of this process have also been developed by various Companies, such as Arkenol.

The separation of the acid for recycling is a critical aspect to improve the process economics.

Lignin is a solid residue of the process, which remains available for various uses, as heat and power generation.

Dilute Acid Hydrolysis. This process dates back to 1800 (first commercial plant in 1898). The dilute acid hydrolysis process first hydrolyses the hemicellulose in mild process conditions (typical process parameters: 0.5-0.7 % H_2SO_4 at 160-190°C) to recover the 5-carbon sugars. The reaction has to be controlled under mild conditions in order to avoid sugar degradation: this not only reduces yields but also causes the formation of inhibitors of the fermentation process (furfurals and other by-products).

Cellulose in the remaining solids is then hydrolysed in a 0.4-2 % H_2SO_4 solution at 200-215°C. As in concentrated acid hydrolysis, solid residues (lignin and residual cellulose) can be used for heat and power generation, while the sugar-rich liquid is sent to fermentation. Ethanol yield (Zimbardi et al. 2002; Hamelinck 2005) is in the range of 74 to 89 % of the theoretical value (89 % for mannose, 82 % for galactose, and only 50 % for glucose).

The reactor design is of particular importance in dilute acid hydrolysis processes, in order to reduce sugar degradation (and so the formation of yeast inhibitors, as furfural), maximise sugar yields and improve the economics of the process.

Enzymatic Hydrolysis. Enzymatic processes, in which selected cellulase enzymes break the polymeric chain of the cellulose and hemicellulose leaving the monomeric sugars available for fermentation, perform a higher hydrolysis yields than chemical processes. However, a barrier for these processes is represented by enzyme costs, that accounts for approximately 40 % of total costs (Zimbardi et al. 2002).

As only 20 % of the biomass pore volume can be reached by the large cellulase enzymes molecules, biomass pre-treatment becomes an essential step in enzymatic hydrolysis processes. In fact, biomass pre-treatment is of primary importance to improve cellulose hydrolysis and therefore increase sugar yields, which are above 90 % with pre-treatment while remain below 20% without pre-treatment. Pre-treatment aims at increasing the surface accessible to enzymes by destroying the cell structure, breaking the

ligninhemicellulose shealth around cellulose and reducing the cellulose polymerisation and crystallinity.

Biomass (Hamelinck et al. 2005) has first to be cleaned (if necessary) and sized (from few centimetres to 1-3 mm).

A cost-efficient pre-treatment stage is a key to the success of the cellulose-to-ethanol process, and a significant R&D effort is today devoted to this area (Ballerini and Monot 2005). Pre-treatment has to be optimised so to increase the yield of cellulose hydrolysis by reducing residence time (i.e. reduced capital costs) as well as enzyme consumption (i.e. reduced operating costs). Moreover, mass losses should be minimised in terms of non fermentable products and fermentation inhibitors.

A list of possible biomass pre-treatment techniques is summarised in the following table.

Table 8.7. Biomass pre-treatments for enzymatic hydrolysis processes (Zimbardi et al. 2002; Hamelinck et al. 2005; Ballerini and Monot 2005).

Biomass	**pre-treatment**			
Method	***Type of process***	***Examples***	***Xylose yield***	***Downstream enzymatic effect (%)***
Physical	Mechanical	Milling Grinding	-	-
	Non mechanical	Irradiation High pressure steaming Liquid Hot Water	- - 88-98 %	- - > 90%
Chemical	Alkali	Dilute NaOH	60-75 %	55
	Oxidative	Oxidative treatment in alcaline peroxide medium	60-75%	50%
	Dilute acid	Dilute sulphuric acid	75-90 %	<85
Physical or Combined Physical-Chemical	Steam explosion (uncatalysed) Steam explosion (catalysed)		45-65 %	90
	Ammonia Fiber Explosion (AFEX) Carbon Dioxide Explosion		-	50-90 (2 steps) 75 (2 steps)
Biological	Enzymes produced by fungi (lab scale)		-	-

Table 8.8. Examples of pre-treatment processes for hydrolysis (Ballerini and Monot 2005).

Physico-chemical	Steam explosion	Iotech, Abengoa, BCI, Sun Opta, CIEMAT, ENEA
	Steam explosion in acid conditions	ASCAF (IFP), Iogen, Un.Lund, ENEA
	Hydrothermal process	Elsam/Sicco
	Wet oxidation	Risoe, Biocentrum-DTU
Chemical	Dilute acid	NREL, ETEK

Energy demand for biomass comminution is indicated in the tale below.

Table 8.9. Energy requirement for mechanical comminution (reprinted from: Cadoche and Lòpez 1989).

Lignocellulosic materials	Final size (mm)	Energy consumption (kWh/ton)	
		Knife mill	Hammer mill
Hardwood	1.60	1.30	130
	2.54	80	120
	3.2	50	115
	6.35	25	95
Straw	1.60	7.5	42
	2.54	6.4	29
Corn stover	1.60	NA[a]	14
	3.20	20	9.6
	6.35	15	NA[a]
	9.5	3.2	NA[a]

[a]NA - not available

Among these processes, steam explosion is probably the most used pre-treatment technique, which can be either uncatalysed (if steam only is used) or catalysed (if chemicals are used). Steam explosion (Zimbardi et al. 2002) consists of feeding a high-pressure reactor with chopped biomass and saturated water steam: biomass is then expelled through a valve. During this process, which can be operated in a batch or continuous mode, the biomass is "exploded" and its main constituents (cellulose, hemicellulose and lignin) separated by the sudden decompression to atmospheric pressure. Steaming times are of the order of seconds/minutes, while typical process parameters are 1.5-4 MPa and 180-230°C. The performances of the steam explosion process depend on the severity of the operating conditions, i.e. temperature and pressure: however, an excessive increase in these parameters promotes the formation of inhibitors of fermentation. Reported xylose-sugars yields are in the range of 45 % - 65 % (Hamelinck et al. 2005). Acid catalysts (as SO_2, which oxidizes at H_2SO_4) can be used for

improving the steam explosion process and increase the overall yield. Steam explosion can be carried out in batch or continuous plants.

Examples of steam explosion plants exist in EU and USA.

Table 8.10. Selected steam explosion plants in EU and USA.

Owner	Location	Capacity	Type	Status
CIEMAT	Spain	200 kg/h	Batch	Existing
Lund University	Sweden	Lab scale	Batch	Existing
ENEA	Italy	300 kg/h		Existing
Stake techn.	Virginia, USA	Lab scale	Bach	Existing
ASCAF	France	2-4 t/h	Continuous	Existing
Abengoa	Babilafuente (Salamanca) Spain	70 t/d	Continuous, Sun-Opta techn.	Contract signed

Liquid Hot Water (LHW) is a further interesting pre-treatment technique for biomass-to-ethanol hydrolysis processes (Hamelinck et al. 2005): it uses compressed hot water, above saturation point, which hydrolyse the hemicellulose and perform a high xylose recovery (88-98 %) without acid or chemical catalyst. LHW is still at laboratory scale.

The main advantages offered by enzymatic hydrolysis are related to the very mild process conditions, that give high yields, and the reduced capital and maintenance costs (reactor materials), as corrosion problems are reduced (Hamelinck et al. 2005).

Since intermediate products (cellobiose and glucose) act as inhibitors of cellulase activity, these have to be removed (by ultrafiltration or by simultaneous fermentation in the same reactor) or extra-enzymes have to be supplied to the reactor. Cellulase represents a significant cost of the enzymatic hydrolysis process, while the main consumable for dilute and concentrated acid hydrolysis is H_2SO_4.

Summarising and comparing (Hamelinck et al. 2005) the three main hydrolysis processes, i.e. dilute acid, concentrated acid, and enzymatic hydrolysis, it can be concluded that:

- the glucose yield vary significantly, from 50-70 % (dilute acid) to 90 % (concentrated acid) to 75 % (enzymatic). Enzymatic hydrolysis promise yields up to 95 % in the future.
- Operating temperatures and residence times are also very different: from 215°C and few minutes (3 min) of dilute acid, to 40°C and 2-6 h of concentrated acid, to 50°C and 1.5 days of enzymatic hydrolysis.

A considerable R&D effort is devoted to process integration. The following main options are today under investigation and development:

- Separate (or Sequential) Hydrolysis and Fermentation, SHF
- Simultaneous Saccharification and Fermentation, SSF
- Simultaneous Saccharification and Co-Fermentation, SSCF
- Consolidated Bio Processing, CBP

Separate (or Sequential) Hydrolysis and Fermentation, SHF. This technique first performs the hydrolysis step converting cellulose into C6 sugars, and then the fermentation step to produce bioethanol. While the positive aspect of this sequential approach is the ability to guarantee optimal process conditions for enzyme and microorganisms (pH, T, oxygen), two distinct reactors are needed, and the inhibition activity of glucose on microorganisms must be taken into account.

Simultaneous Saccharification and Fermentation, SSF. Saccharification and fermentation are carried out simultaneously in a single reactor, thus allowing for cost saving and reduction of inhibitors, increasing hydrolysis rate. Obviously, the optimisation of process conditions for both enzymens and microorganisms at the same time is the critical issue of this solution.

Simultaneous Saccharification and Co-Fermentation, SSCF. Continuous alcoholic co-fermentation of hexose and pentose sugars in a coimmobilized system configuration containing cultures of *S.cerevisiae* and *C.shehatae*.

Consolidated Bio Processing, CBP. All enzymes and bioethanol are produced in a single reactor by a single microorganisms community.

Process costs are expected to significantly decrease from SSF (10.5 €/GJ) to SSCF (9.8 €/GJ) to CPB (4.5 €/GJ). A detailed description of these processes is available in literature (as Hamelinck et al. 2005; Zimbardi et al. 2002; Zacchi 2005).

3.2.2 Thermochemical conversion processes

A different approach to bioethanol production from lignocellulosic biomass is represented by the thermochemical path, which consists of biomass gasification followed by catalysed reaction or fermentation. In fact, in both cases, the first stage of the process is the production of a low calorific value gas, which is then cleaned, and either catalytically synthesized to the final product or fermented.

Three main steps characterise of the first method (Caraballo 2005):

1. *biomass gasification*, in which syngas is produced (and cleaned) from solid biomass. Gasification is the thermochemical conversion of biomass at high temperature (~ 800°C), in the presence of an oxidising agent (as air, steam or oxygen), into a low calorific value raw gas, steam and tar.
2. *syngas transformation*, where gas composition is adjusted by catalytic synthesis processes
3. *separation of products*

Main advantage of the thermochemical processes is the capability to use the entire biomass as well as different feedstocks, but gas cleaning is still a major issue to make this solution sufficiently efficient and economically viable. The products of these processes are also called BTL (Biomass To Liquid) fuels, as liquid fuels are produced from solid (lignocellulosic) biomass.

The synthesis of ethanol and other alcohols from syngas is a very important part of the process. In particular, ethanol synthesis is carried out similarly to Fisher Tropsch or methanol production, but with lower results and therefore overall efficiency. In order to increase bioethanol yield, more R&D effort has to be directed to the catalyst field.

Table 8.11. Main reactions for ethanol and higher alcohol synthesis (Caraballo 2005).

Methanol synthesis	$CO + 2\ H_2 \rightarrow CH_3OH$
Water shift reaction	$CO + H_2O \rightarrow CO_2 + H_2$
Ethanol synthesis	$2\ CO + 4\ H_2 \rightarrow C_2H_5OH + H_2O$
Higher alcohol synthesis	$n\ CO + 2n\ H_2 \rightarrow C_nH_{(2n+1)}OH + (n-1)\ H_2O$
Olefins formation	$n\ CO + 2n\ H_2 \rightarrow C_nH_{2n}OH + n\ H_2O$
Paraffins formation	$n\ CO + 2\ (n+1)\ H_2 \rightarrow C_nH_{(2n+2)}OH + n\ H_2O$
Synthesis of other products	DME, methyl ester, acetic acid, etc
Product homologation	

The H_2/CO ratio has to be optimised to maximise the product yield (approximately 1 for ethanol synthesis), as well as process parameters such as pressure (typically > 50 bar), temperature (~ 240-350°C), and the type of catalyst.

Another possible option for bioethanol production via mixed thermochemical-biological path is represented by syngas fermentation (Spath and Dayton 2003). Similarly to the above reported BTL route, the initial stage is again biomass gasification: the CO and H_2 rich syngas is then fermented to bioethanol, which is recovered from the broth with processes similar to

those typical of the corn industry, as distillation and adsorption. The cell mass can be recycled to the gasifier, while it is not approved as animal feed.

As for the previous case, a significant advantage offered by the adoption of a first gasification stage is that a wide range of low-cost biomass feedstocks can be used, thus increasing biofuel production potential and reducing feedstock costs.

The fermentation reactor can be a simple gas-sparged reactor (either batch or continuously operated), but other designs (as two-stage reactors) with better performances have been studied and proposed (Klasson et al. 1991). Weak aspects of the gas-sparged reactor are low volumetric productivity, low gas conversion and very diluted bioethanol production (up to 2% v/v).

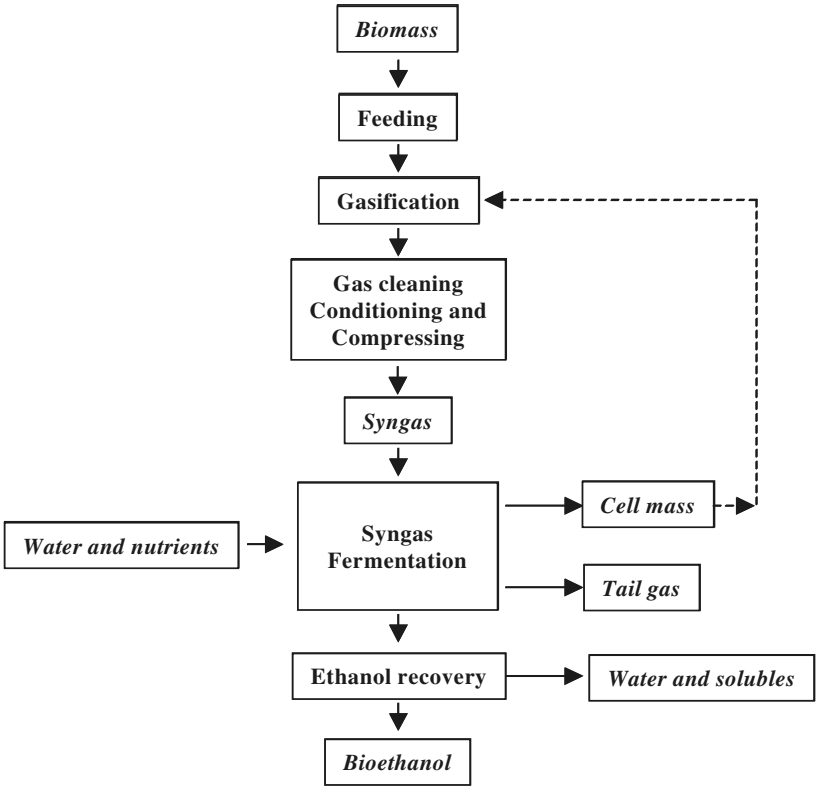


Fig 8.11. Bioethanol production via syngas fermentation (modified from Spath and Dayton 2003).

4. Bioethanol use as transport fuel

4.1 Ethanol/ETBE in spark ignition engines

Ethanol can be used in different ways as engine fuel. Non-food bioethanol is always denatured by adding a small amount of foreign materials which makes it unsuitable for human consumption. Blends of 5 % v/v (E5) anhydrous bioethanol in gasoline do not require any modification to standard engines or to the distribution infrastructures. Bioethanol is blended in gasoline up to 10 % v/v (E10) in USA and even up to 22 % v/v (E22) in Brazil, a major bioethanol producer. Brazil bioethanol use in cars dates back from 1975 for gasohol, blends of gasoline and anhydrous alcohol and 1979 for hydrated ethanol (neat ethanol cars) (Moreira 2005).

The use of higher blends of bioethanol in gasoline is also possible but the technology has to be adapted to meet the different physical-chemical characteristics of the biofuel. 85 % v/v (E85) is used in Flexible Fuel Vehicles: FFVs represent a main innovation in the car market, as these vehicles are able to recognise any blend from 0 % to 85 % and consequently adapt the main engine parameters. Neat hydrous ethanol can be used in dedicated E95 Internal Combustion Engine Vehicles (ICEV), or in Fuel cell vehicles with on-board reforming.

Table 8.12. Neat and blended bioethanol fuels and use in different Countries (Schieder 2005; Specht 2005).

Neat/blended biofuel	Description	Use	Countries
ETBE	<15 % v/v blend in gasoline	All gasoline engines	Spain, Italy, Germany, France
E5	5 % v/v ethanol in gasoline		Any country
E10 (Gasohol)	10 % v/v ethanol in gasoline	All gasoline engines in USA	Sweden, Canada, USA, Brazil
E22	22 % v/v ethanol in gasoline	All gasoline engines in Brazil	Brazil
E85	85 % v/v ethanol in gasoline	Flexible Fuel Vehicles (FFV)	Brazil, USA, Sweden
E95	95 % v/v ethanol in gasoline	Dedicated engines	Sweden, Brazil
E100	100 % ethanol		
EtOH in Diesel (Diesehol) + Additive	< 15 % v/v ethanol in diesel	Almost standard diesel engine	Sweden, USA

The ethanol's low volatility limits the ignition capacity of bioethanol at low temperature (cold start): so, without blending with gasoline, the issue of wintertime start must be carefully addressed by engine manufacturers. Ethanol is also used for ETBE $CH_3CH_2OC(CH_3)_3$ production. ETBE (Ethyl-Tertiary-Butyl-Ether) is an oxygenated additive for gasoline made from ethanol (47 %) and % isobutylene (53 %), which can be blended to gasoline up to 15 % vol (known as ETBE/15G). Properties of ETBE are summarised and compared to both MTBE and ethanol in the next table.

Table 8.13. ETBE properties (Source: Blondy 2005).

Properties	Euro Super specif.(1)		ETBE	MTBE	Ethanol
	2002/2004	2005			
Sulfur (ppm)	150	50/10	< 10	< 10	< 1
Benzene (% vol)	1	1	0	0	0
Aromatics (% vol)	42	35	0	0	0
Olefins (% vol)	18	18	0	0	0
Oxygen (weight)	2.7	2.7	14.3	16.3	34.8
RON	95/98	95/98	108-112	106-110	120-130
MON	85/87	85/87	96-100	93-97	96-100
Vapour pressure (kPa)	60	60(2)	45	65	200
Low calorific value (kj/I)	31200		27150	26150	21250

(1) Maximum values, except octane
(2) Summer values

ETBE has a low volatility and a low aromatic content, thus reducing Volatile Organic Compound emissions. Its high octane number makes this oxygenated additive particularly suitable for high efficiency engines, in which the compression ratio is higher. ETBE is totally compatible with existing cars and infrastructures.

4.2 Ethanol in compression ignition (diesel) engines

4.2.1 Ethanol in Diesel (Diesehol)

Bioethanol in diesel oil (up to 15 % v/v) and almost neat ethanol fuel (i.e bioethanol with 10 % w/w additives) in diesel engines are also under investigation and used in some Countries, especially in Sweden.

The introduction of bioethanol in diesel oil seems a promising and effective mean to improve the environmental performances of the engine, especially in terms of smoke emission reduction: it is studied since the 1970's (South Africa). However, the properties of bioethanol are critical to its use in diesel: in particular, the reduction in the fuel cetane number (bioethanol cetane number is ~8, while typical values for diesel oil are around 48), the increased ignition delay, and the stability of the ethanol-diesel mixture (which can separate at temperature below room temperature) require the use of additives. The changes in other important fuel characteristics, as viscosity, lubricity and heating value, must also be properly addressed, together with the variation in Flash Point and Vapour Pressure.

Bioethanol can be added to diesel oil at different levels (Mc Cormick et al. 2001; Satgé de Caro 2001):

- Emulsions in the range of 5-15 % v/v of ethanol are technically possible (by adding a limited amount of organic polyfunctional additives) and economically interesting.
- 20-40 % v/v emulsions are possible, but the amount of additive needed per litre is considerable.
- The use of almost neat (90 %) ethanol in diesel is also technically possible (see next chapter).

Other possible means for using bioethanol in diesel engines are fumigation and dual injection (Chiaramonti and Tondi 2004), not discussed here because of minor interest for future applications.

Further than increasing the cetane number (which decreases linearly with ethanol content) and improving lubricity, emulsifiers are needed to stabilise the emulsion, to make the emulsion tolerant towards water and to increase material compatibility. They must be used also in case of very low amount (5 % v/v) of ethanol in diesel. Biodiesel is a good emulsifier for bioethanol in diesel fuels.

As regards the performances of engines with e-diesel (ethanol-diesel blends, up to 15 % v/v), recent experimental work (Dominguez et al. 2005) on off-road (2-cylinder, air cooled, mechanical direct injection) and light-duty

(4-cylinder, water cooled, supercharged, common rail) engines gave the following main results:

- Mechanical direct injection engines are sensitive to bioethanol addition (increased injection delay), while electronically (common rail) controlled ones are rather indifferent to bioethanol addition.
- A modest power output loss (5 % at 15 % v/v) was observed, due to the lower heating value of bioethanol compared to gasoline, as well as an increase in the brake specific fuel consumption
- Smoke opacity was lowered at higher bioethanol blends, while no clear conclusions were achieved as regards NOx and particulates. At low loads, instead, increase in HC and CO emissions was observed.

Transport, handling, storage and safety of diesel-ethanol mixtures are a major issue compared to diesel oil. In fact, the flammability limit of e-diesel is very close to pure ethanol, which is ~50°C lower than pure diesel and ~30°C lower than pure gasoline. Specific measures, norms and standards for ethanol-diesel blends have to be developed and applied in transportation and storage, since these e-diesel blends must be handled as gasoline and not as diesel oil.

4.2.2 Neat ethanol in diesel engines

Use of neat ethanol in diesel has been developed and widely tested in Sweden.

Sekab has developed and is commercialising a special bioethanol-based fuel for the use in large urban buses (ETAMAX-D). Specifications for ETAMAX-D are given in the table below (www.sekab.se; Wästljung 2005).

Table 8.14. ETAMAX-D main physical-chemical characteristics (from www.sekab.se).

Appearance		Clear, without particles	ASTM D 2090
PH		min 5.2, max 9.0	AMSE 1131
Water	% w/w	max 6.2	SS-ISO 760
Density (D 20/4)	g/ml	0.82 - 0.84	SS-ISP 758
Fuel Composition			
Ethanol 95 %	% w/w	90.2	
Ignition improver	% w/w	7.0	
MTBE	% w/w	2.3	
Isobutanol	% w/w	0.5	
Corrosive inhibitor	ppm	90	
Colour		Red	

MTBE (Methyl Tert-Butyl Ether) and iso-butanol are used as denaturants, ignition improver is Beraid 3540. Corrosion inhibitor is up to 125 ppm. SCANIA has been testing ethanol in diesel buses since 1985 in Sweden: today, 434 buses have been sold to 13 cities in Sweden. The main adaptation of city-bus engines are the following:

- Increase in compression ratio (24:1 / 18:1)
- Enlargement of nozzles (to allow for a larger fuel flow)
- Modification of the injection timing
- Installation of larger fuel tanks and increase in fuel pump flow
- Change of gaskets and filters

Stockholm (Ljung 2005) today has the largest EU bus fleet (253 buses in 2004, 132 new buses expected to run in 2005-2006) running with bioethanol: all inner city buses are fed with ethanol.

The environmental benefits associated with the use of neat bioethanol in diesel engines are considerable. NOx are reduced at 56 % of those typical of EURO 2 diesel engines, CO to 3.2-1 % of Euro 2 standard as well as HC (8-13 % of Euro 2 standard).

Table 8.15. Material compatibility with ethanol and ethanol-blended fuels (from Renewable Fuel Association 2002).

RECOMMENDED	NOT RECOMMENDED
metals	
Aluminium	Zinc-galvanized (ethanol only)
Carbon steel	
Stainless steel	
Bronze	
Elastomers	
Buna-N (hoses & gaskets) [(1)]	Buna-N (seals only) [(1)]
Fluorel [(1)]	Neoprene
Fluorosilicone [(2)]	Urethane rubber
Neoprene (hoses & gaskets)	
Polysulfide rubber	
Natural rubber (ethanol only)	
Viton [(1)]	
Polymers	
Acetal	Polyuretane [(2)]
Nylon [(2)]	Alcohol-based pipe dope (recently applied)
Polypropylene	
Teflon [(1)]	
Fiberglass reinforced plastic [(2)]	

[(1)] Registered trademark; [(2)] The manufacturer of the specific material should be consulted

The present status of ethanol engines is able to meet the Euro 4 standard (Wästljung 2005). CO emissions are 1.5 g/kWh, hydrocarbon 0.46 g/kWh, NOx 3.5 g/kWh, and particulates 0.02 g/kWh.

4.4 Material compatibility

When using bioethanol in engines, a particular attention has to be given to verify that engine and storage tank materials are compatible with this corrosive fuel. In fact, materials for tanks, pumps, sealants, filters, and other components have to be selected according to their characteristics: specifications on material compatibility with bioethanol are available.

Long-term tests (2000 hours) have been conducted on non-automotive engines to verify material compatibility with 10 and 20 % ethanol in gasoline (Orbital 2003). Metal, brass and polymeric materials were investigated. Corrosion of several metallic parts normally exposed to fuel was reported: care has to be given to those parts where the oxides could dislodge and become trapped between moving parts, thus accelerating component wear-out. All brass components showed to be tarnished, indicating that oxidation was occurring: this can affect fuel metering and control in carburetors. Finally, some polymeric components (as fuel line connectors, delivery hoses, bulbs, etc) were also significantly affected by the contact with ethanol: this cannot be accepted, as it can cause fuel leakage.

A number of guidebooks and information documents are available as regards handling, storing and dispensing ethanol fuels (Renewable Fuel Association 2002; Center for Transportation Research 2000).

4.4 Industries and biofuels

The growing interest in biofuels production and use is stimulating discussions among biofuel producers and end-user, namely car industries and oil Companies. They have recently expressed their point of view on biofuels and, in particular, on bioethanol.

Volkswagen AG (Seyfried 2005) seems in favour of 5 % v/v bioethanol blending (which should be possible to increase up to 10 % v/v without major efforts, while lower EtOH blending should be avoided). In addition, according to VW, E15-E20 blending in GDI (Gasoline Direct Injection) engines still need some R&D work, and significantly higher blending (as E85, in FFVs) presents higher risks of phase separation, require dedicated infrastructures for fuel distribution, and could be problematic as far as concerns cold start behaviour.

Abengoa is implementing a large project on bioethanol, in Spain.

Other industrial actors, as some EU oil Companies (e.g. Total, Blondy 2005), seems instead more interested in BtL (Biomass to Liquid) fuels (as Sunfuel), rather than on bioethanol.

5. Economics

Bioethanol production costs have been estimated and assessed by various authors, among which Hamelinck (2003 currency). A summary of Hamelinck's figures is given in the following table.

Table 8.16. Bioethanol production costs from different feedstocks and technologies (Hamelinck et al. 2005).

Feedstock type	Bioethanol cost	Country
Sugar cane	10-12 €/GJ_{HHV}	Brazil
Sugar/starch crops	16.2-23 €/GJ_{LHV}	USA, Europe
Lignocellulosic biom.	34-45 €/GJ_{HHV}	Europe
Lignocellulosic biom.	15-19 €/GJ_{HHV}	USA

Hamelinck also reports forecasts by various authors about future projected bioethanol costs, that range from 4.5-10 €/GJ_{HHV}, to 6-8 €/GJ_{HHV}, to 10-11 €/GJ_{HHV}.

Traditional bioethanol production in Brazil is today competitive with gasoline. The main reasons for bioethanol success in the Brazilian context (Moreira 2005) relies in the following three main factors:

- The external debt, which is decreased thanks to the savings generated by ethanol production and use
- The available land: 18 % increase of total harvested area in the period 1988-2004
- The low cost for manpower

These production costs have however been reached after a long development work of the entire chain, which lasted more than 20 years, as reported by Goldemberg.

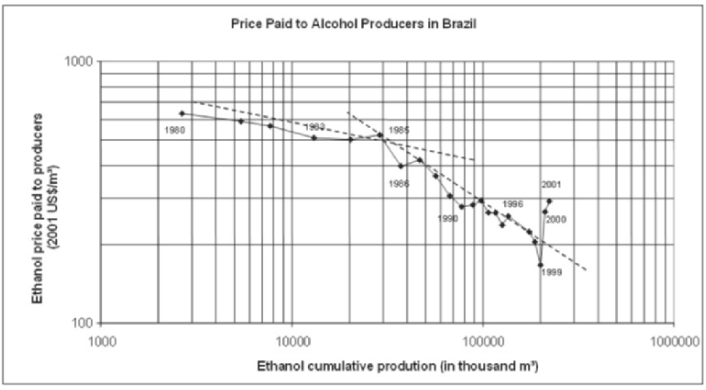


Fig. 8.12. Ethanol learning curve, Brazil (source: Goldemberg. Avail.at: http://www.treckin.com/seminarwssd/goldemberg.ppt)

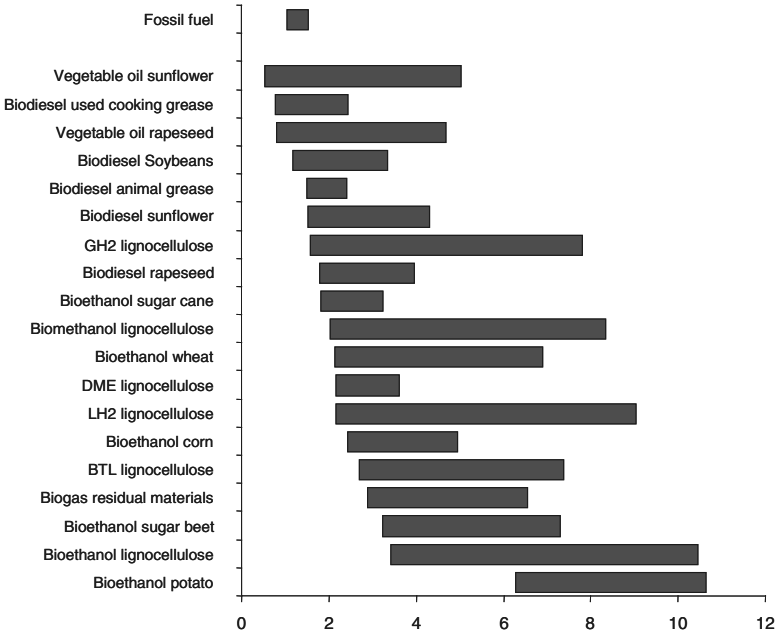


Fig. 8.13. Biofuel supply costs in €/100 km. (Source: IFEU).

IFEU also analysed various biofuels and related supply cost estimations. Main results are summarised in the following figure.

6. Environmental aspects and sustainable production in EU

The environmental aspects of bioethanol production and use is a major and very complex issue to be analysed, as it involves a proper understanding of the whole chain and the availability of a large amount of information. LCA or "well-to-wheels" studies must then be developed in order to achieve reasonable estimations. Deep investigations on GHG (Greenhouse gas) emission reduction, energy input/output ratio etc., have been carried out by various authors, and a considerable discussion is on going on these issues. Reviews are on this subject available in literature (e.g. IEA 2004).

The analysis of GHG emissions and energy balance of bioethanol must be carried out distinguishing between:

- Ethanol from grains
- Ethanol from sugar beets
- Ethanol from sugar cane
- Ethanol from lignocellulosic feedstocks

Ethanol from grains. A summary by the International Energy Agency (IEA) of the results of a large number of studies is reported in the following table.

As reported above, almost all studies except the one by Pimentel (2001) conclude that a GHG emission reduction in the range 20-40 % can be expected. The review of net-energy studies on bioethanol developed by the US Dept. for Agriculture (Shapouri et al. 2002) indicates that the key issues for these analysis are:

- Corn yield per hectare.
- Ethanol conversion efficiency and energy requirements.
- Energy embedded in the fertiliser used to grow corn.
- Assumptions regarding use of irrigation.
- The value, or "energy credit", given for co-products produced along with ethanol (mainly animal feed).

These factors varies across the studies reviewed by Shapouri: in particular, Pimentel (2001) seems not to take into account the recent (last 10 years) improvements in crop yields and conversion efficiencies, and includes some factors (such as the energy embedded in farm equipment and the cement used in bioethanol plant construction) which however accounts only for a small part of the differences.

Table 8.17. Energy and GHG Impacts of Ethanol from grain: estimates from Corn- and Wheat-to-Ethanol Studies (reprinted from IEA 2004).

	Feedstock	Ethanol production efficiency (litres/tonne feedstock)	Fuel process energy efficiency (energy in /out)	Well-to-wheels GHG emissions: compared to base (gasoline) vehicle (per km travelled)	
				Fraction of base vehicle	Percent reduction
GM/ANL 2001	corn-a	372.8	0.50	n/a	n/a
GM/ANL 2001	corn-b	417.6	0.55	n/a	n/a
Pimentel 2001/91	corn	384.8	1.65	1.30	-30%[c]
Levelton 2000	corn	470.0	0.67	0.62	38%
Wang 2001a	corn-dry mill	387.7	0.54	0.68	32%
Wang 2001a	corn-wet mill	372.8	0.57	0.75	25%
Levy 1993	corn-a	367.1	0.85	0.67	33%
Levy 1993	corn-b	366.4	0.95	0.70	30%
Marland 1991	corn	372.8	0.78	0.79	21%
Levington 2000	wheat	348.9	0.90	0.71	29%
ETSU 1996	wheat	346.5	0.98	0.53	47%
European Commission 1994	wheat	385.4	1.03	0.81	19%
Levy 1993	wheat-a	349.0	0.81	0.68	32%
Levy 1993	wheat-b	348.8	0.81	0.65	35%

Note: Where a range of estimates is reported by a paper, "*a*"and "*b*" are shown in the feedstock column to reflect this. [c] Negative greenhouse gas reduction estimate connoted and increase. n/a: not available. Sources: Except for Levelton 2000, Wang 2001a and GM/ANL 2001, data presented here for these studies are taken from the comparison conducted by CONCAWE 2002.

IEA reports that recent evaluations estimate that one energy unit of bioethanol requires 0.6-0.8 fossil energy unit. It is important to remark that most of this energy is not petroleum-based: Shapouri estimates only 17 % from oil fuels, the rest from natural gas and coal, thus making only 0.12-0.15 energy units of petroleum-based fuels necessary to produce one unit of bioethanol from grains (or, alternatively, "one gasoline-equivalent litre of bioethanol displaces 0.85-0.88 liters of petroleum on a net energy basis"-IEA, 2004).

Ethanol from sugar beet. IEA indicates that GHG emission reduction could be up to 56 %, but also that (1) the same factors presented above for

grains also apply to this feedstock, and that (2) more work is needed for a more detailed assessment which reduces the large variations in feedstock production and conversion efficiencies.

Ethanol from sugar cane. The use of fossil energy in bioethanol production from sugar cane is considerably lower than the previous cases, thanks to (1) the rather high yields of sugar cane under low fertilisation conditions and (2) the use of bagasse for power and heat to feed the bioethanol production process. The net energy balance (energy output/energy input) of bioethanol production have been widely studied over the last decades, and main findings report an energy gain between 0.56 and 0.84. Nevertheless, more recent work by Macedo et al. 2003, concluded that the net energy balance ranges between 8 and 10: it means that per each energy unit of bioethanol, only 0.1 unit of fossil energy are needed. These results are possible thanks to the significant improvement in sugar cane yields per hectare as well as in conversion processes, which have been achieved in the period 1985-2002.

As a consequence, well-to-wheel CO_2 emission reduction is estimated at 92 %, well above previous estimations by various authors (ranging between 35 % and 56 %).

Table 8.18. Energy balance of sugar-cane-to-ethanol in Brazil in 2002 (Reprinted from IEA 2004. Source: Macedo et al. 2003).

	Energy requirement (MJ/tonne of processed cane)	
	Average	**Best values**
Sugar cane production	**202**	**192**
Agricultural operations	38	38
cane transportation	43	36
Fertilisers	66	63
Lime, herbicides, etc	19	19
Seeds	6	6
Equipment	29	29
Ethanol production	**49**	**40**
Electricity	0	0
Chemicals and lubricants	6	6
Buildings	12	9
Equipment	31	24
Total energy input	**251**	**232**
Energy output	**2089**	**2367**
Ethanol	1921	2051
Bagasse surplus	169	316
Net energy balance (out/in)	**8.3**	**10.2**

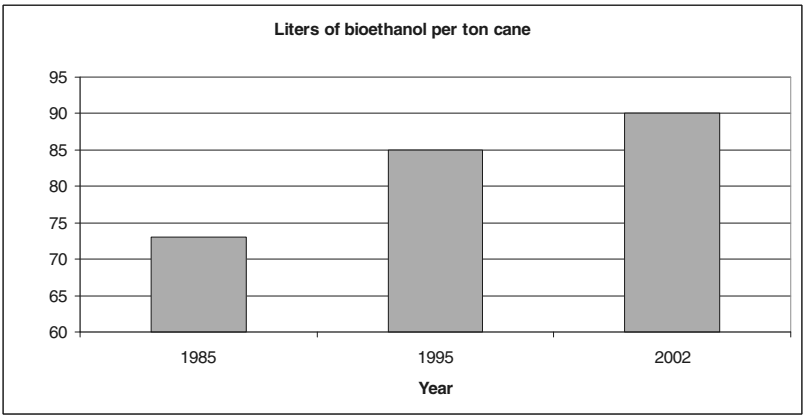


Fig. 8.14. Bioethanol production in Brazil (Source: Macedo et al. 2003).

Ethanol from sugar lignocellulosic biomass. The process efficiency as well as the well-to-wheel emission of bioethanol from lignocellulosic biomass has been evaluated by various authors: main differences are due to variations in end-use vehicle efficiency and assumptions on fertiliser needs. Average estimations are around 70-90 %.

Table 8.19. Energy and GHG Impacts of Ethanol from lignocellulosic: Estimates from Corn- and Wheat-to-Ethanol Studies (Reprinted from IEA 2004).

	Feedstock	**Ethanol production efficiency (litres/tonne feedstock)**	**Fuel process energy efficiency (energy in /out)**	**Well-to-wheels GHG emissions: compared to base (gasoline) vehicle (per km travelled)**	
				Fraction of base vehicle	Percent reduction
GM e*t al*. 2002	wood (poplar plantation)	n/a	1.20	0.49	51%
GM/ANL 2001	wood-*a*	288	1.30	n/a	n/a
GM/ANL 2001	wood-*b*	371	1.90	n/a	n/a
Wang 2001a	wood	288	1.52	-0.07	107%
GM/ANL 2001	grass-*a*	303	100	0.29	71%
GM/ANL 2001	grass-*b*	390	1.60	0.34	66%
Wang 2001a	grass	303	1.37	0.27	73%
Levelton 2000b	grass	310	1.28	0.29	71%
GM et a.l 2002	crop residue (straw)	N/a	n/a	0.18	82%

Table 8.19. Energy and GHG Impacts of Ethanol from lignocellulosic: Estimates from Corn- and Wheat-to-Ethanol Studies (Reprinted from IEA 2004).

	Feedstock	Ethanol production efficiency (litres/tonne feedstock)	Fuel process energy efficiency (energy in /out)	Well-to-wheels GHG emissions: compared to base (gasoline) vehicle (per km travelled)	
Levelton 2000b	corn residue (stover)	345	1.10	0.39	61%
Levelton 2000	hay	305	1.32	0.32	68%
Levelton 2000	wheat straw	303	1.12	0.43	57%

Note: Where a range of estimates is reported by a paper, "*a*" and "*b*" are shown in the feedstock column to reflect this.

n/a: not available

[a]Process energy includes both biomass and non-biomass energy sources.

Sources: GM *et al*. (2002), GM/ANL *et al*. (2001), Wang (2001a), and Levelton (2000b).

Considering non-GHG emissions, two types of emissions must be considered, i.e. exhaust and evaporative ones.

The use of bioethanol blended in gasoline at 5-7 % v/v is able to reduce CO emissions by 14-40 %, and Unburned Hydrocarbons (UHC) emissions are the same or lower (by 2-7 %) of gasoline-fuelled vehicles. Also particulate emissions benefit from bioethanol blends. Slightly increase or no change in NOx emissions are instead observed. Aldehyde emissions (chemical compounds associated with incomplete combustion of bioethanol) are reduced by the high efficiency of catalytic converters used in ethanol-cars.

Evaporative emissions relate to fuel evaporation and release in the atmosphere from vehicle fuel tank. Considering E85, it has fewer highly volatile components than gasoline, and therefore lower evaporative emissions. However, it also results into difficult ignition at low temperature (possible cold start problems for the engine).

As regards the EU region and its potential for environmentally sustainable biofuel production, the EEA (European Environmental Agency) recently carried out a wide analysis on bioenergy potential from agriculture, forest, and waste in 2010, 2020, 2030 (Wiesenthal 2005). Taking into account that the use of biomass must not generate additional pressure on farmland, forest biodiversity, and soil and water resources, and that all other environmental constraints must be respected, as regards energy crops the analysis achieved the conclusion reported in the following figure (see also EEA briefing 02-2005, ISSN 1830-2246).

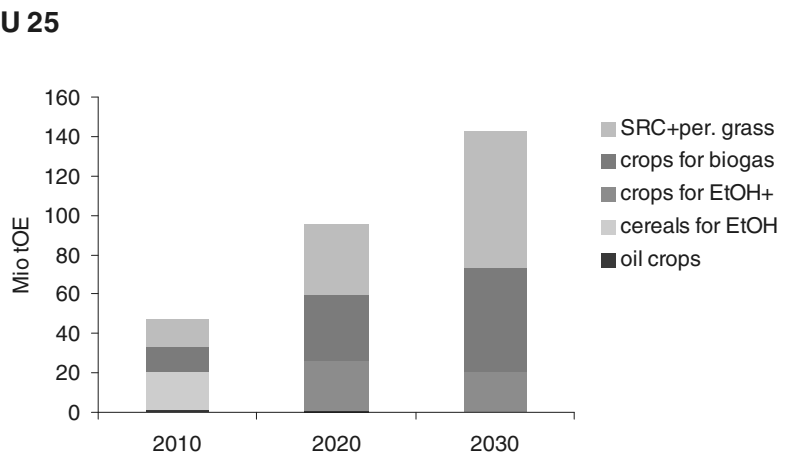


Fig. 8.15. Environmentally compatible bioenergy potential from agriculture by energy crops (Source: Eur. Envir. Agency – EEA, Wiesenthal 2005).

According to the EEA analysis, the potential offered by 1st generation biofuels seems limited in dimension: in particular, sugar beet and rapeseed oil do not look so favourable in the EU-25. However, as cereals generally have lower impact than sugar beet and better yield the rapeseed, in the short term traditional bioethanol from cereals seems more promising than biodiesel. In the medium-long term new technologies are expected to enter into the market (2nd generation biofuels), which are instead more promising from the environmental point of view, as they can use the whole crop (lignocellulosic material, including by-products from agriculture). Moreover, feedstock is less expensive that traditional oil/sugar crops.

Finally, the use of bioethanol-derived oxygenated additives, as ETBE and ethers, has a number of environmental advantages, such as reduction of carbon monoxide emissions, reduction in the aromatic content of gasoline and resulting toxics, reduction of olefin content of gasoline, reduction of volatile organic compounds emissions (precursors of ozone pollution), and reduction of carbon dioxide.

In the BIOFIT project ("Bioenergy for Europe: which ones best fit? – A comparative analysis for the community", available at www.ifeu.de) a comprehensive investigation based on ISO 14040-14043 standards (LCA analysis) has been carried out. Among various solid and liquid biofuels, ETBE from sugar beet has been evaluated and compared to MTBE (for France, Germany and The Netherlands). The use of ETBE offers advantages as regards greenhouse effect and reduction of fossil fuel use, while the impact in terms of acidification and eutrophication is not positive. The

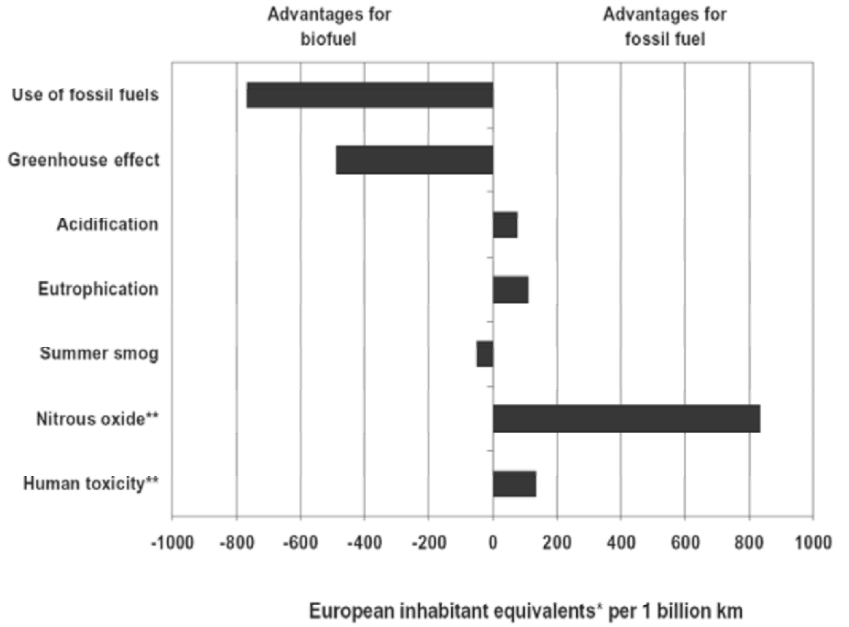


Fig. 8.16. ETBE from sugar beet versus MTBE (Source: IFEU, BIOFIT Final Report, available at www.ifeu.de)

effect on summer smog is instead almost equivalent. It has to be remarked that this study considered three EU Countries and sugar beet only.

7. Conclusions

Bioethanol is already the main actor worldwide in the biofuel sector, and its role is expected to steadily grow in the coming years. In fact, market estimations forecast a rapid increase in bioethanol production and use even in those regions of the world (such as Europe) where bioethanol is still lagging behind biodiesel. The development and industrialisation of hydrolysis technologies for bioethanol production from lignocellulosic biomass are expected to be the key issue for this development.

The potential for environmentally sustainable bioethanol production from lignocellulosic biomass is significantly greater than traditional production from sugar or starch crops. In addition, environmental benefits are higher as well.

The constant quality of bioethanol as transport fuel makes possible its use as neat or blended fuel in a wide range of engines, from spark to compression ignition ones.

Finally, the recent revision of the Common Agricultural Policy, together with the ambitious targets set by the European Commission in the framework of the Greenhouse Gas emission reduction measures, will boost the production and use of this biofuel in the next years in the EU.

Acknowledgments

The author wish to acknowledge Prof.Ing. Francesco Martelli, Dr. Francesco Zimbardi, Ing. Giacobbe Braccio, Dr. Tobias Wiesenthal and Ing. Aldo Nardi, for their contributions, suggestions and revision of the present work.

References

Blondy J (2005) Biofuels in Europe for the next coming years. In: Sjunnesson L, Carrasco J, Grassi A, Helm P (eds) Proceedings of the 14th European Biomass Conference. Paris

Bryden Kenneth M. (1989) Computational modeling of wood combustion. University of Winsconsin – Madison

Cadoche L, Lòpez GD, (1989) Assessment of size reduction as a preliminary step in the production of ethanol from lignocellulosic wastes. Biol.Wastes 30: 153-157

Caraballo J (2005) Ethanol synthesis. In: Stoehr M (ed) 1st European Summer School on Renewable Motor Fuels. Birkenfeld

Center for Transportation Research, Energy System Division, Argonne National Laboratory (2000) Guidebook for Handling, Storing & Dispensing of Fuel Ethanol. Prepared for the US Department of Energy

Chiaramonti D, Tondi G (2004) Stationary Application of Liquid Biofuels". Final Report EC PTA Contract nr NNE5-PTA-2002-006, Avail.at http://europa.eu.int/comm/energy/res/sectors/bioenergy_publications_en.htm

CTI (2002) Riduzione dell'Impatto Ambientale dei Motori Diesel, Milano

DG XII (1994) Application of biologically derived products as fuels or additives in combustion engines. EUR 15647 EN

Dominguez JI, Miguel E, Millàn C, Arjona R (2005) The effects of ethanol-diesel blended fuels on the performance and emissions of unmodified engines. In: Sjunnesson L, Carrasco J, Grassi A., Helm P (eds) Proceedings of the 14th European Biomass Conference. Paris

European Commission (2005) Biomass Action Plan. COM(2005) 628 Final

European Commission (2005)Impact Assessment. Annex to the Biomass Action Plan, COM(2005) 1573

Gaddy JL, Clausen, EC (1992) "*Clostridium ljungdahlii*, An Anaerobic Ethanol And Acetate Producing Microorganism", US Patent 5173429

Gaddy JL, Clausen EC, Ko CW, Wade L.E, Wikstrom CV (2002) Microbial Process For The Preparation Of Acetic Acid As Well As Solvent For Its Extraction From The Fermentation Broth, US Patent 6368819 B1

Grassi G and Nillson L (2005) Innovative technology from Bioethanol Production. In: Sjunnesson L, Carrasco J, Grassi A., Helm P (eds) Proceedings of the 14th European Biomass Conference. Paris

Fulton L (2004) Biofuels for transport: an international perspective. Available at www.ictsd.org.

Hamelinck CN, Faaij APC (2005) Outlook for advanced biofuels. Energy Policy, in press

Hamelinck CN, Van Hooijdonk G, Faaij APC (2005) Ethanol production from lignocellulosic biomass: techno/economic performance in short-, middle- and long-term. Biomass and Bioenergy 28: 384-410.

http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/C/Carbohydrates.html

http://www.agra-net.com/portal/

http://www.energies-renouvelables.org/

http://www.sekab.se

http://www.treckin.com/seminarwssd/goldemberg.ppt

IEA. (2004) Biofuels for Transports - An International Perspective. Available at www.iea.org.

Klasson KT, Ackerson MD, Clausen EC, Gaddy JL (1991) Biorector Design for Synthesis Gas Fermentations. Fuel, vol 70, p 605-614

Lackmann J (2005) The political framework in Germany for biofuels in transport. In: Sjunnesson L, Carrasco J, Grassi A., Helm P (eds) Proceedings of the 14th European Biomass Conference. Paris

Ljung M (2005) Towards sustainable travel in Stockholm's public transport. In: Sjunnesson L, Carrasco J, Grassi A., Helm P (eds) Proceedings of the 14th European Biomass Conference. Paris

Macedo IC et al. (2003) "Greenhouse Gas Emissions in the Production and Use of Ethanol in Brazil: Present Situation (2002)", prepared for the Secretariat of the Environment, São Paulo

Mc Cormick R, Parish R (2001) Advanced Petroleum Based Fuels Program and Renewable Diesel Program – Milestone Report: Technical Barriers to the Use of Ethanol in Diesel Fuel. NREL. Contract No DE-AC36-99-GO10337

Moreira JR (2005) Overview of bioenergy activity for transport in Brazil. In: Sjunnesson L, Carrasco J, Grassi A., Helm P. (eds) Proceedings of the 14th European Biomass Conference. Paris

Nallerini D, Monot F (2005) Biological conversion of lignocellulosic biomass: present and future of biofuels production. In: Sjunnesson L, Carrasco J, Grassi A., Helm P (eds) Proceedings of the 14th European Biomass Conference. Paris

Neij (2005) Prospects of cost reduction for lingo-cellulosic ethanol in Sweden – A methodological approach. In: Sjunnesson L, Carrasco J, Grassi A., Helm P (eds) Proceedings of the 14th European Biomass Conference. Paris

Orbital Engine Company (2003) Market Barriers to the Uptake of Biofuel Study – A Testing Based Assessment to Determine Impact of 10 % and 20 % Ethanol Gasoline Fuel Blend on Non-Automotive Engines – 2000hrs Material Compatibility Testing. Orbital Engine Company, Report to Environment, Australia

Piacente E, Walter A Bio-ethanol trade: Opportunities and challenges for Brazil. In: Sjunnesson L, Carrasco J, Grassi A., Helm P (eds) Proceedings of the 14th European Biomass Conference. Paris

Renewable Fuel Association (2002) Fuel Ethanol - Industry Guidelines, Specification and Procedures. RFA Publication nr 960501

Satgé de Caro P, Mouloungui Z, Vaitilingom G, Berge JCh (2001) Interest of combining an additive with diesel-ethanol blends for use in diesel engines. Elsevier, Fuel 80: 565-574

Schieder D (2005) Bioethanol - Existing pathways. In: Stoehr M (ed) 1st European Summer School on Renewable Motor Fuels. Birkenfeld

Seyfried F (2005) Biomass for transport – the message of a leading European Car Company (VW). In: Sjunnesson L, Carrasco J, Grassi A., Helm P (eds) Proceedings of the 14th European Biomass Conference. Paris

Shapouri H, Duffield JA, Wang M (2002) The energy balance of corn ethanol: an update. Agricultural Economic Report nr 813

Spath PL, Dayton DC (2003) Preliminary screening – Technical and economic assessment of synthesis gas to fuels and chemicals with emphasis on the potential for biomass-derived syngas. NREL Technical Report, NREL/TP-510-34929. Available at http://www.osti.gov/bridge

Specht M (2005). Why biofuels? – An Introduction into the Topic. In: Stoehr M (ed) 1st European Summer School on Renewable Motor Fuels. Birkenfeld

Sun Y and Cheng J (2002 Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresource Technology 83: 1-11

Van Loo S and Koppejan J (eds) (2002) Handbook of Biomass Combustion and Co-Firing. Twente University Press, Enschede, ISBN 9036517737

Wästljung U (2005) Biofuels for road transport: twenty years of experience of ethanol fuelled buses. In: Sjunnesson L, Carrasco J, Grassi A., Helm P (eds) Proceedings of the 14th European Biomass Conference. Paris

Wiesenthal T (2005) Environmental considerations for bioenergy production. In: Sjunnesson L, Carrasco J, Grassi A, Helm P (eds) Proceedings of the 14th European Biomass Conference. Paris

Zacchi G (2005) Fuel ethanol production from lignocellulosic via simultaneous saccharification and fermentation – technical and economic challenges. In: Sjunnesson L, Carrasco J, Grassi A, Helm P (eds) Proceedings of the 14th European Biomass Conference. Paris

Zimbardi F, Viola E, Gallifuoco A, De Bari I, Cantarella M, Barisano D, Braccio G (2002) Overview of the bioethanol production. Report ENEA and University of L'Aquila in the framework of the EU project "Production of clean hydrogen from fuel cells by reformation of bioethanol". Available at www.cti2000.it

9

Complex lipid biosynthesis and its manipulation in plants

Irina A. Guschina, John L. Harwood

School of Biosciences, Cardiff University, Museum Avenue, CF10 3US, Wales, UK (e-mail: harwood@cf.ac.uk)

1. Introduction

In all living organisms lipids play several roles and, according to their structures, can be divided into two main groups: the non-polar lipids (acylglycerols, sterols, free fatty acids, wax and steryl esters) and polar lipids (phosphoglycerides, glycosylglycerides, and sphingolipids). Triacylglycerols act as compact, easily metabolised and non-hydrated energy stores. They are important storage products especially in plants producing oilseeds and in oily fruits such as avocado, olive and oil palm. Waxes are commonly extracellular components such as surface coverings, which function both to reduce water loss and to protect plants from noxious environmental conditions. They also act as an energy store in jojoba.

Polar lipids and sterols are important structural components of cell membranes with many diverse functions. The membrane lipids act as permeability barriers for cells and organelles (Gurr et al. 2002). They provide the matrix for assembly and function of a wide variety of catalytic processes as well as directly participating in metabolism and in a multitude of membrane fusion events. Moreover, the membrane lipids actively influence the functional properties of membrane-associated processes (Gurr et al. 2002). In additional to a structural role, lipids act as key intermediates

P. Ranalli (ed.), Improvement of Crop Plants for Industrial End Uses, 253–279.

in cell signalling pathways (e.g. inositol lipids, sphingolipids, oxidative products) and play a role in sensing changes in the environment.

Plant fats and oils are utilised for many food and industrial applications. They include edible oils, processed ingredients for the food industry and feedstocks for chemical processes such as formulation of paints, inks, resins, varnishes, plasticizers and biodiesel production (Kridl 1998).

Over the last decades, research into plant lipid metabolic pathways has expanded considerably and it has been influenced by an increasing impact arising from molecular genetic approaches. Many genes encoding lipid-related enzymes have been isolated/cloned and this has allowed the manipulation of plant lipid metabolism for commercial purposes (Slabas and Sanda 1998; Murphy 2005). In theory, this genetic engineering approach required cloning of the genes controlling certain steps of biosynthesis, regulating the genes for proper expression in the seeds of plants and a transformation and regeneration system for the oil-seed of choice (Kridl 1998). In this chapter we will summarize how this approach has been used for oil improvements and modulations of lipid biosynthesis.

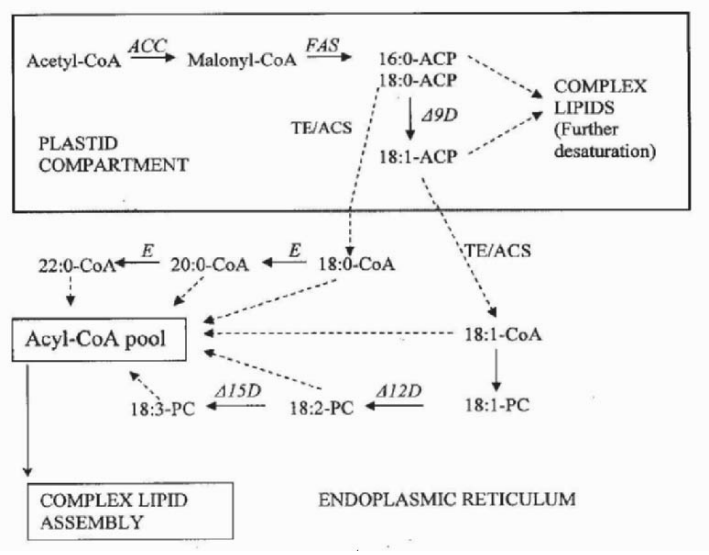


Fig. 9.1. Simplified depiction of fatty acid biosynthesis in plants. Fatty acids are abbreviated with the number before the colon indicating the number of carbon atoms and the number afterwards showing the number of double bonds. Thus 16:0 = palmitic acid, 18:1 = octadecenoic acid (oleic acid in this case) etc. Abbreviations: *ACC*, acetyl-CoA carboxylase; *FAS*, fatty acid synthase; *D*, desaturase (e.g. *Δ9D*, Δ9-desaturase); *E*, fatty acid elongase; TE, acyl-ACP thioesterase; ACS, acyl-CoA synthase.

2. Summary of lipid metabolism

Before summarising molecular biological attempts to manipulate lipid metabolism in plants we must write a few words about the pathways concerned. A detailed discussion is not appropriate here and the reader is referred to Gurr et al. (2002) for a simple description and Murphy (2005) for more details. Appropriate references will be found in these books.

Basically, de novo synthesis uses acetyl-CoA carboxylase and fatty acid synthase to produce palmitic and stearic acids. The latter can be further modified by desaturation and elongation reactions and these processes use enzymes in different compartments of plant cells (Fig. 9.1).

Complex lipids, usually based on a glycerol backbone, are made by the basic Kennedy pathway, together with additional reactions (Fig. 9.2).

3. Fatty acid manipulation

Fatty acid biosynthesis is one of the primary pathways of lipid metabolism and an exclusive source of the acyl chains of complex lipids (Harwood 1996; 2005). The major fatty acids from the world oil supply (mainly from soybean, palm and canola) are palmitic, linoleic and oleic acids (Hildebrand et al. 2005). Many unusual fatty acids have been identified in seed oils as major components. In some cases, these unusual acids comprise more than 90% of the seed oil (Hildebrand et al. 2005). Fatty acids may differ in terms of their chain length, degree of saturation, configuration of double bonds, positional isomers, conjugation of double bonds or additional chemical groups such as hydroxy, allenic, epoxy, acetylenic, cyclo, fluoro and keto. The discovery of many biological activities (as well as industrial applications) of some unusual fatty acids has led to an increased utilization of these molecules and to an attempt to domesticate several unusual fatty acid-producing oilseeds. Since domestication of most of these unusual oilseeds faces a number of diverse problems (e.g. low yield, climatic requirement), genetic engineering approaches often seem to be a more promising route for the development of such resources (Hildebrand et al. 2005).

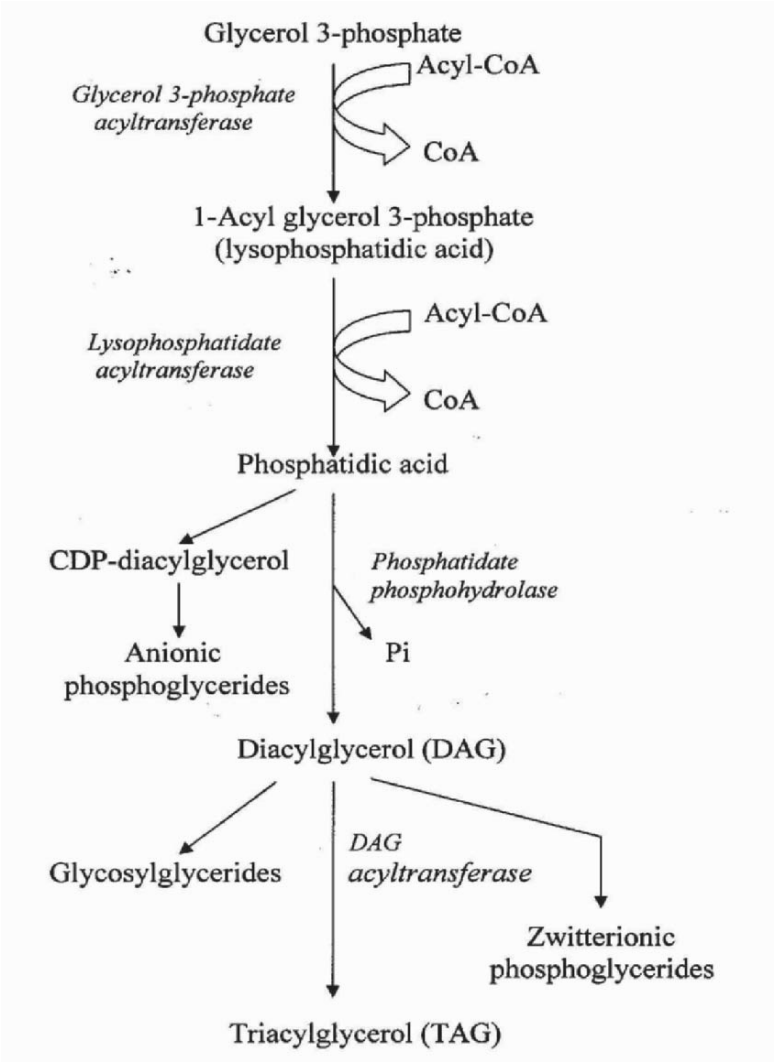


Fig. 9.2. The basic Kennedy pathway for glycerolipid synthesis in plants. Important anionic phosphoglycerides in plants are phosphatidylglycerol, phosphatidylinositol and diphosphatidylglycerol (cardiolipin). Zwitterionic phosphoglycerides include phosphatidylethanolamine and phosphatidylcholine. For additional reactions that can be involved in phosphoglyceride and triacylglycerol formation, refer to Dörmann (2005) and Weselake (2005).

Plants synthesize 18-carbon fatty acids through a pathway located in the plastid that begins with acetyl-CoA and then uses malonyl-acyl carrier protein (ACP) as the two-carbon donor (Fig. 9.1). The acyl-CoA needed for this synthesis comes ultimately from photosynthesis. The actual process of de novo synthesis to produce long-chain fatty acids involves the participation of two enzymes, acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) which are usually multi-protein complexes containing a number of enzymes. ACC is a soluble Class 1 biotin-containing enzyme that catalyses the ATP-dependent formation of malonyl-CoA from bicarbonate and acetyl-CoA. This malonyl-CoA is used for de novo synthesis of fatty acids inside plastids. In addition, malonyl-CoA is needed for elongation of fatty acids on the endoplasmic reticulum as well as for synthesis of various secondary metabolites in the cytosol. As expected from such requirements, two isoforms of ACC are found in plants, the second of which is extrachloroplastic (presumed to be cytosolic) and is a multifunctional protein. These isoforms have distinct properties which give rise to their different susceptibility to herbicides (Harwood 2005).

FAS is the second major enzyme complex involved in de novo fatty acid formation. The plant FAS is a type II dissociable multiprotein complex, like the *E. coli* system but unlike that of animals, i.e. the individual proteins that make up FAS can be isolated and their function demonstrated separately. The first condensation reaction in fatty acid synthesis is catalysed by β-ketoacyl-ACP synthase III (KAS III) that uses acetyl-CoA and malonyl-ACP substrates to give a 4C-keto-intermediate. Successive reduction, dehydration, and a second reduction then produce a 4C fatty acid, butyrate, which is attached to ACP. The next six condensations are catalysed by KAS I to produce 6-16C fatty acids. The final reaction between palmitoyl-ACP and malonyl-ACP uses KAS II and results in synthesis of stearate. The remaining enzymes of FAS are β-ketoacyl-ACP reductase, β-hydroxylacyl-ACP dehydrase and enoyl-ACP reductase (Fig. 9.3).

Completion of de novo fatty acid synthesis is made in one of three ways. Either the product of acyl-ACP is hydrolysed by a thioesterase, the palmitate or stearate products are desaturated or the acyl-ACPs are used directly for complex lipid formation using plastid acyltransferases. Through the action of thioesterases, non-esterified fatty acids are made available for conversion to acyl-CoAs which provide the acyl chains for assembly into complex lipids in the extra-plastid compartment (Fig. 9.1).

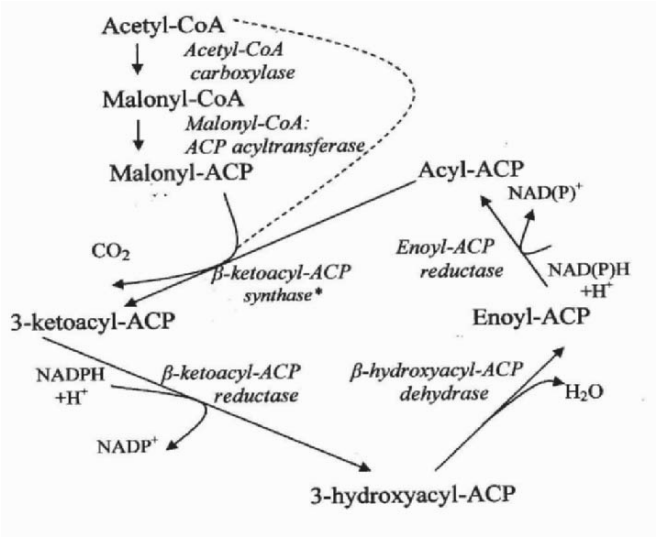


Fig. 9.3. Reactions of fatty acid synthase. *The first condensation reaction is catalysed by β-ketoacyl-ACP synthase III (KAS III) that uses acetyl-CoA and malonyl-ACP substrates. The next six condensations are calalysed by KAS I and the final reaction between palmitoyl-ACP and malonyl-ACP uses KAS II.

3.1 β-ketoacyl-ACP reductase modification

This enzyme has been isolated and purified from several plant tissues and the monomer from *Brassica napus* has a molecular mass of 28 kDa (Slabas et al. 1992). cDNAs have been isolated from a number of plants and show 55-81% identity for their derived sequences (Harwood 1996). The *Arabidopsis* library shows only one gene for β-ketoacyl-ACP reductase so far identified (Mekhedov et al. 2000). The *B. napus* β-ketoacyl-ACP reductase was down-regulated using an antisense approach (O'Hara et al. 2000). Only during the rapid phase of leaf expansion (days 4-7 after emergence) was it thought that β-ketoacyl-ACP reductase could contribute to any significant effects on total fatty acid synthesis rates. At other times, the major control of flux appeared to reside elsewhere in the pathway. Nevertheless, seeds gathered from antisensed *Brassica* lines showed some distinct morphologies and a reduced lipid content (O'Hara et al. 2000).

3.2 β-ketoacyl-ACP synthase modification

Expression of cDNA encoding KAS III showed that this enzyme has a universal role in fatty acid biosynthesis, irrespective of the plant species from which it is derived or the tissue in which it is expressed (Dehesh et al. 2001). An increase in the levels of C16:0 was observed in tobacco (*Nicotiana tabacum,* WT-SR) leaves overexpressing KAS III from spinach (*Spinacia oleracea*) when under the control of the cauliflower mosaic virus-35S promoter and in arabidopsis and rapeseed (*Brassica napus*) seeds overexpressing either of the *Cuphea hookeriana* KAS IIIs when expression was driven by napin. The transgenic seeds contained lower levels of oil as compared with the wild-type levels. In addition, the rate of lipid synthesis in transgenic rapeseed seeds was notably slower than that of the wild-type (Dehesh et al. 2001). The levels of the acyl-ACP intermediates as well as any changes in levels of other fatty acid synthase enzymes have been measured and it was suggested that malonyl-ACP, the carbon donor utilised by all the 3-ketoacyl-ACP synthases, was limiting in the transgenic plants. Malonyl-CoA has been further suggested to be a potential limiting factor affecting the final oil content and C16:0 extension (Dehesh et al. 2001).

In order to understand the contribution to chain length regulation that might be made by β-ketoacyl-ACP synthase, Cw KAS A1, derived from *Cuphea wrightii* (a species that accumulates 30% C10:0 and 54% C12:0 in its seed oil) was investigated by Leonard et al. (1998). Expression of this gene in *Arabidopsis* seeds, especially when combined with *C. wrightii* thioesterase (which has good activity with medium chain fatty acids), allowed a doubling of the production of C10:0 (Leonard et al. 1998).

Decreased amounts of C18:1 and increased amounts of C18:2 and C18:3 acids were observed as compared to control plants when *E. coli fabH* gene, which encodes a KAS III-equivalent enzyme, was overexpressed in *B. napus* (Verwoert et al. 1995). Thus, expression of KAS genes cannot always be predicted to give a simple change in fatty acid composition.

3.3 Acyl-ACP thioesterase modification

Since medium-chain fatty acids are valuable renewable resources, attempts have been made by researchers to produce high-yielding annual crops using genetic transformation. Expression of FatB1 cDNA encoding 12:0-acyl-ACP thioesterase (BTE), isolated from the seeds of undomesticated California bay, in the seeds of *Arabidopsis thaliana* and *Brassica napus* resulted in BTE activity, and in the accumulation of medium chain fatty

acids at the expense of long-chain FAs (Voelker et al. 1992; Yuan et al. 1995). Laurate became the most abundant FA and was deposited in the storage lipids (up to 60% of the triacylglycerol acyl groups) indicating that the "foreign" fatty acid was exported from the plastids and utilised by the enzymes of the Kennedy pathway for the assembly of triacylglycerides (TAGs) (Voelker et al. 1992). Non-destructive analysis of the oil composition of single seeds enabled high-lauric lines to be selected from the best events and, through subsequent breeding and performance trials, a cultivar has been developed. This cultivar is known as "Laurate Canola" (Laurical™) and a very acceptable yield of oil was obtained from seeds grown in Michigan in the summer of 1995 and a crop in North Dakota in November 1996. The oil is being used for the manufacture of soaps and detergents (Kridl 1998; Davies 1996). The possibility of Laurical™ use as a food ingredient is also under investigation.

Production of high levels of caprylate (C8:0) and caprate (C10:0) has also been achieved in transgenic canola, which normally does not accumulate any short chain FAs, by overexpression of Ch FatB2, a thioesterase cDNA isolated from the Mexican shrub *Cuphea hookeriana* (Dehesh et al. 1996). A dramatic increase in the levels of these two fatty acids was accompanied by a preferential decrease in the levels of linoleate and linolenate (Dehesh et al. 1996).

Eccleston and co-workers transformed *B. napus* using a medium-chain acyl-ACP thioesterase isolated from *Umbellularia californica* (California bay). Although laurate accumulated in seeds, none was detectable in leaves despite very high levels of the medium-chain thioesterase. If this thioesterase was expressed in seeds using a napin promoter, then up to 60% of the total seed fatty acids were laurate. At the same time β-oxidation was increased and this was believed to be responsible for the limited accumulation of seed laurate when the constitutive CaMV 35S promoter was used. In support of this hypothesis, isocitrate lyase activity was found to be significantly increased in plants transformed with FatB1. In the high-laurate seeds, the levels of acyl carrier protein and several enzymes of fatty acid synthesis were increased, perhaps to compensate for the lauric acid lost through β-oxidation (Eccleston et al. 1996; Eccleston and Ohlrogge 1998).

The tropical tree species mangosteen (*Garcinia mangostana*) has been found to store stearate (C18:0) in its seed oil in amounts of up to 56% by weight (Hawkins and Kridl 1998). Expression of mangosteen thioesterase (Garm FatA1) in *Brassica* seeds led to the accumulation of stearate up to 22% in seed oil suggesting that Garm FatA1 is, at least, partially responsible for determining the high stearate composition of mangosteen seed oil (Hawkins and Kridl 1998).

3.4 β-ketoacyl-CoA synthase modification

This enzyme catalyzes the condensation of malonyl-CoA with long-chain acyl-CoA and this reaction is the initial step of the microsomal fatty acyl-CoA elongation pathway responsible for formation of very long chain fatty acids (VLCFAs, fatty acids with chain length > 18 carbons) (Fig. 9.1). Manipulation of this pathway is significant for agriculture, because this was the basis for the conversion of high erucic acid (C22:1) rapeseed (HEAR) into canola cultivars. Although canola varieties are used for food purposes, there is an interest in HEAR cultivars to provide lubricant oils. TAGs from HEAR varieties of rapeseed lack erucoyl residues in the sn-2 position, and there has been considerable interest in raising the erucate levels further by overcoming this compositional limitation (Davies 1996). This objective has been approached in the following way by Lassner et al. (1996). First, they cloned a gene involved in an elongation reaction, β-ketoacyl-CoA synthase (KCS), from the jojoba plant, *Simmondsia chinensis.* In its native species this enzyme is part of the "elongase" system that produces the C20, C22 and C24 acyl groups that predominate in the stored wax esters. The introduction of this gene into canola resulted in the production of TAGs containing up to 58% of VLCFAs. The KCS gene will now be used to isolate the homologous gene from HEAR, in order to overexpress it and, thus, obtain an oil containing higher than the typical HEAR value of 40-50% erucate (Davies 1996).

From *Limnanthes douglasii*, cDNAs that encoded a homolog of KCS involved in production of VLCFA synthesis in this species have been isolated (Cahoon et al. 2000). Expression of FAE1 homolog in somatic soybean embryos showed the accumulation of C20 and C22 fatty acids, principally as eicosanoic acid, up to levels of 18% (w/w) of the total fatty acids of single embryos.

An 8-fold increase in erucic acid proportion in *Arabidopsis* seed oil has also been seen as a result of seed-specific expression of nasturtium (*Tropaeolum majus*) KCS (or elongase) gene (Mietkiewska et al. 2004).

3.5 β-ketoacyl-CoA reductase modification

Metz and co-workers purified an alcohol-forming fatty acyl-CoA reductase (FAR) from developing embryos of the jojoba, *S. chinensis*, plants (Metz et al. 2000). Jojoba is known to produce another kind of reserve lipid in seeds, namely esters of long-chain alcohols and fatty acids (waxes), which have an important use in cosmetics. When the jojoba FAR cDNA was expressed in embryos of *B. napus*, long-chain alcohols could be detected in

transmethylated seed oils showing that approximately 4% of the acyl groups were reduced to alcohol groups. It is interesting that, in addition to free alcohols, novel wax esters were identified in the transgenic seed oil (Metz et al. 2000). An endogenous fatty acyl-CoA: fatty alcohol acyltransferase activity, that could account for this wax synthesis, has been identified using *B. napus*. Thus, introduction of a single cDNA into *B. napus* resulted in a redirection of a portion of seed oil synthesis from TAGs to waxes (Metz et al. 2000). Lardizabal et al. (2000) combined these two cDNAs with a KCS elongase cDNA from *Lunaria annua* (*Brassicaceae*) for coexpression in *A. thaliana* under control of a napin promoter. In some seeds, as much as 70% of the oil was accounted for by wax and the proportion of VLCFAs and alcohols in some of these seeds was increased to 42% as compared to 28% in the wild type (Lardizabal et al. 2000).

3.6 Desaturase manipulation

18:1-Δ12-desaturase and related enzymes

The greatest functional diversity within a family of fatty-acid-modifying enzymes has been observed within the Δ12-oleic acid desaturase or Fatty Acid Desaturase 2 (FAD2) family (Jaworski and Cahoon 2002). This enzyme, which is typically found in nearly all higher plants, catalyzes the insertion of a *cis* double bond between the Δ12 and Δ13 carbon atoms of oleic acid (C18:1-Δ9) to form linoleic acid (C18:2-Δ9,12). By downregulation of FAD2 (which blocked the flux from oleic acid into polyunsaturated fatty acids) in soybean, lines with 85% of oleic acid in seed oil have been produced (Kinney 1998a). Cotton transgenic lines transformed with the microsomal ω-6 (Δ12-) desaturase ghFAD2-1 inverted-repeat construct have been shown to exhibit the increased level of oleic acid from about 15% in untransformed cotton plants up to a range of 26-77% in the mature seeds of primary transgenic plants (Liu et al. 2000). Chapman and coworkers also reported the development of transgenic cotton plants with higher seed oleic acid contents (Chapman et al. 2001). A binary vector was designed to suppress expression of the endogenous cottonseed Δ12 desaturase (FAD2) by subcloning a mutant allele of a rapeseed FAD2 gene downstream from a heterologous, seed-specific promoter (phaseolin). Increased seed oleic acid content ranged from 21 to 30% (by weight) of total fatty acids and was at the expense of linoleic acid, consistent with reduced activity of cottonseed FAD2. The integration of the canola transgene into the cotton genome has been confirmed by molecular analysis of nuclear DNA from transgenics (Chapman et al. 2001).

Co-suppression plasmids carrying oleate desaturase genes from each species have been constructed and transferred into Australian elite breeding lines of *B. napus* and *B. juncea* using *Agrobacterium tumifaciens* plant-transformation techniques (Stoutjesdijk et al. 2000). Silencing of the endogenous oleate desaturase genes resulted in substantial increases in oleic acid levels, of up to 89% in *B. napus* and 73% in *B. juncea* (Stoutjesdijk et al. 2000).

Some plants have evolved new functions for their FAD2 and many variant forms of this enzyme have been identified, e.g. hydroxylases, epoxygenases, acetylenases and conjugases (Fig. 9.4).

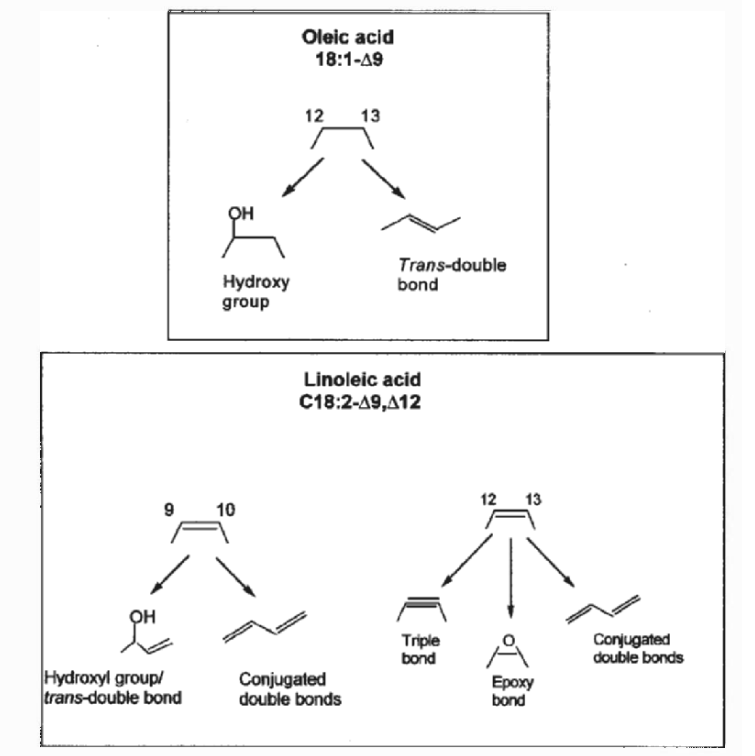


Fig. 9.4. Summary of reactions catalyzed by FAD2-related enzymes (see text for further details).

Analysis of the phylogenetic relationships of acetylenase, conjugase, epoxygenase, hydroxylase and desaturase amino acid sequences indicated that acetylenases, conjugases, epoxygenases and hydroxylases group semi-randomly among Δ12 desaturase sequences suggesting that these enzymes

arose independently many times from pre-existing desaturases during plant evolution (Hildebrand et al. 2005).

The Δ12-oleic acid hydroxylase from the seeds of castor (*Ricinus communis*) was described as the first divergent form of FAD2 (Van de Loo et al. 1995). This enzyme introduces a hydroxyl group at the Δ12 position of oleic acid to produce the industrially valuable ricinoleic acid (12-OH-18:1-Δ9). Hydroxy fatty acids from castor seeds can be used in a wide range of commercial products including plastics, foams, surfactants, cosmetics and lubricants (Jaworski and Cahoon 2003; Kinney and Clemente 2005). Oleate 12-hydroxylase genes have been cloned from developing endosperm of the castor-oil plant and *Lesquerella fendleri* (Brassicaceae) (Van de Loo et al. 1995; Broun and Somerville, 1997; Broun et al. 1998). A cDNA encoding the oleate 12-hydroxylase from castor bean has been shown to have approximately 67% sequence homology to microsomal oleate desaturase from *Arabidopsis* and to direct the synthesis of small amounts of ricinoleic acid in seeds of transgenic tobacco plants (Van de Loo et al. 1995). Expression of the cDNA under control of a napin promoter in transgenic *Arabidopsis thaliana* plants resulted in the accumulation of up to 17% of seed fatty acids as ricinoleate and two novel fatty acids, lesquerolic (14-OH-20:1-Δ11) and densipolic (12-OH-18:2-Δ9,Δ15) acids (Broun and Somerville 1997). These results suggested that, either the castor hydroxylase can utilize oleic acid and eicosenoic acid for substrates for ricinoleic and lesquerolic acid biosynthesis, respectively, or that *Arabidopsis* contains an elongase that accepts ricinoleic acid as a substrate. The discovery of a condensing enzyme from the seeds of *L. fendleri* that specifically elongated hydroxyl fatty acids supported the latter suggestion (Moon et al. 2001). It is also interesting, that expression of the castor bean oleate 12-hydroxylase in *A. thaliana* led to a concomitant increase in oleic acid in the seed oil, from 14.7% to 24.1% (Broun and Somerville 1997). Expression of the *L. fendleri* oleate 12-hydroxylase in transgenic plants of a FAD2 mutant of *Arabidopsis,* which was deficient in cytoplasmic oleate Δ12 desaturase activity, resulted in partial suppression of the mutant phenotype in roots (Broun et al. 1998a). Thus, both hydroxylase and desaturase activities have been suggested for the *L. fendleri* enzyme (Broun et al. 1998a). Moreover, expression in yeast indicated that the castor hydroxylase itself has a low level of desaturase activity (Smith et al. 2000).

Partitioning between desaturation and hydroxylation activities has been achieved by exchanging the identity of amino acids at four key locations within the *A. thaliana* FAD2 and the *L. fendleri* hydroxylase/desaturase (Broun et al. 1998b). Broadwater and co-workers reported that four analogous substitutions in the FAD2 sequence by their equivalents from the castor oleate hydroxylase resulted in hydroxy fatty acid accumulation in *A.*

thaliana to the same levels as for the wild-type castor hydroxylase (Broadwater et al. 2002). Interestingly, control experiments showed that the wild-type *A. thaliana* FAD2 desaturase has inherent, low level, hydroxylation activity. Moreover, fatty acid desaturases from different kingdoms and with different regiospecificities exhibited similar intrinsic hydroxylase activity, underscoring the fundamental mechanistic similarities between desaturation and hydroxylation (Broadwater et al. 2002).

A cDNA (Cpal1) encoding a Δ12-epoxygenase that can catalyse the synthesis of 12,13-epoxy-18:1-Δ9 (C18:1E, vernolic acid) acid from linoleic acid has been isolated from *Crepis palaestina* (Asteraceae) (Lee et al. 1998; Singh et al. 2001). When the Cpal1 gene was expressed under the control of a napin promoter in *A. thaliana*, the seed lipids accumulated only low levels (6.2% of the total fatty acids) of C18:1E and also 12,13-epoxy-18:2-Δ9,Δ15 (C18:2E) acid. The level of oleic acid was increased significantly in these plants, whereas the levels of linoleic and linolenic acids were decreased indicating that endogenous Δ12-desaturation was greatly reduced. Coexpression of a Δ12-desaturase from *C. palaestina* in Cpal2 transgenic *Arabidopsis* returned the relative proportion of C18 seed fatty acids to normal levels and resulted in an almost twofold increase in total epoxy fatty acids (Singh et al. 2001). Seed oil of *Stokesia laevis* has been shown to contain 60-70% vernolic acid and an epoxygenase gene has been cloned from *S. laevis* and expressed in *Arabidopsis* (Hatanaka et al. 2004). The average content of vernolic acid in seeds of these transgenic *Arabidopsis* plants was 5.8 μg/mg dry weight (2.4% of total fatty acids on average) (Hatanaka et al. 2004).

In contrast to *C. palaestina*, results from metabolic experiments suggested the involvement of a cytochrome P450 enzyme in vernolic acid synthesis in seeds of the Euphorbiaceae species *Euphorbia lagascae* (Bafor et al. 1993). An expressed sequence tag strategy has been successfully used by Cahoon and co-workers to identify a cytochrome P450 cDNA (designated *CYP726A1*) that corresponded to a gene that was highly expressed in *E. lagascae* seeds (Cahoon et al. 2002). Expression of this cDNA in tobacco callus and somatic soybean embryos resulted in the production of Δ12-epoxy fatty acids (C18:1E and C18:2E) at up to 15% and 8% (w/w) of the total fatty acids of these transgenic tissues, respectively (Cahoon et al. 2002).

The results described above illustrate opportunities for the production of industrially-valuable epoxy fatty acids in transgenic oilseeds. Furthermore, applications of acids such as vernolic acid as plasticizers of polyvinyl chloride, adhesives, coating materials and a precursor of monomeric components of nylon-11 and nylon-12 have already been demonstrated (Cahoon et al. 2002).

Vegetable oils that contain fatty acids with conjugated double bonds, such as tung oil, are valuable drying agents in paints, varnishes and inks. Cahoon and co-workers first demonstrated the ability of transgenic plants (e.g. somatic soybean embryos) to produce fatty acid components of these oils when they expressed conjugase cDNAs isolated from developing seeds of *Momordica charantia* and *Impatiens balsamina*. These species accumulate large amounts (as much as 17% w/w of the total fatty acids) of α-eleostearic (C18:3-Δ9*cis*, Δ11*trans*, Δ13*trans*) and α-parinaric acids (C18:4-Δ9*cis*,Δ11*trans*,Δ13*trans*, Δ15*cis*), respectively (Cahoon et al. 1999). A class of FAD-related enzymes that modified Δ9-double bonds to produce the conjugated *trans*, *trans*-Δ10-double bonds found in calendic acid (C18:3-Δ8*trans*, Δ10*trans,* Δ12*cis*) from the seed oil of *Calendula officinalis* have been studied by Cahoon et al. (2001). In somatic soybean embryos expressing these genes, calendic acid accumulated at up to 22% (w/w) of the total fatty acids.

A gene encoding a bifunctional fatty acid Δ12 conjugase/desaturase has been cloned from the tung (*Aleurites fordii*) tree (Dyer et al. 2002). The cDNAs that encoded a class of conjugases, associated with the formation of *trans*-Δ11, *cis*-Δ13 double bonds, have also been isolated from *Trichosanthes kirilowii* and *Punica granatum* (Hornung et al. 2002; Iwabuchi et al. 2003). Expression of these genes in *Arabidopsis* seeds under transcriptional control of a napin promoter showed the accumulation of punicic acid (C18:3-Δ9*cis*, Δ11*trans*, Δ13*cis*) at up to 10% (w/w) of the total seed oil (Iwabuchi et al. 2003). It is interesting, that the conjugase was also found to be bifunctional and exhibited Δ12-oleate desaturase activity (Iwabuchi et al. 2003).

Cahoon and Kinney used an expressed sequence tag (EST) analysis of developing *Dimorphotheca sinuate* seeds to provide direct evidence for the biosynthetic origin of dimorphecolic acid (9-OH-C18:2-Δ10*trans*, Δ12*trans*) which is unusual in containing a C-9 hydroxyl group, Δ10,Δ12-conjugated double bond and *trans*-Δ12 unsaturation (Cahoon and Kinney 2004). EST analysis revealed the occurrence of two structurally divergent forms of FAD2 in *D. sinuate* seeds that were designated DsFAD2-1 and DsFAD2-2. Expression of DsFAD2-1 in soybean somatic embryos resulted in the accumulation of the *trans*-Δ12 isomer of linoleic acid (C18:2-Δ9*cis*,Δ12*trans*) rather than the more typical *cis*-Δ12 isomer. When coexpressed with DsFAD2-1 in soybean embryos, DsFAD2-2 converted C18:2-Δ9*cis*,Δ12*trans* into dimorphecolic acid. When DsFAD2-2 was expressed alone in soybean embryos or together with a typical *cis*-Δ12-oleic acid desaturase in yeast, trace amounts of the *cis*-Δ12-isomer of dimorphecolic acid were formed from *cis*-Δ12-linoleic acid (Cahoon and Kinney 2004).

Recently, production of *trans*-10, *cis*-12 conjugated linoleic acid has been reported in tobacco seeds and rice after transformation with the linoleate isomerase gene from *Propionibacterium acnes* (Hornung et al. 2005; Kohno-Murase et al. 2006). Although the amount of this conjugated acid was relatively low (e.g. up to 1.3% w/w of the total fatty acids in the seeds of transgenic rice), these results demonstrated the potential ability of this simple genetic transformation for the production of such a conjugated fatty acid which has been shown to have a number of biological effects (e.g. anti-carcinogenic and anti-atherosclerosis) (Kohno-Murase et al. 2006).

In *Crepis rubra*, oleate is a substrate in the synthesis of the acetylenic acid, 9-octadecen-12-ynoic acid (crepenynic acid). Lee et al. (1998) characterized an enzyme involved in the synthesis of this acid in *C. alpina* (Lee et al. 1998). When the acetylenase gene was expressed in *Arabidopsis*, total fatty acids from seeds of individual T_0 transgenic plants contained up to 25% (w/w) crepenynic acid in contrast to control plants where it was not found.

18:0-Δ9-ACP-desaturase

This enzyme catalyzes the first desaturation step in plant lipid and seed oil biosynthesis, converting stearoyl-ACP to oleoyl-ACP (Fig. 9.1). Seed-specific antisense gene constructs of *B. rapa* stearoyl-ACP desaturase were used to reduce the protein concentration and enzyme activity of steroyl-ACP desaturase in developing rapeseed embryos during storage lipid biosynthesis (Knutzon et al. 1992). The resulting transgenic plants showed dramatically increased stearate levels (up to 45%) in the seeds of transgenic *B. napus* plants (Knutzon et al. 1992). The similar technique of stearoyl-ACP downregulation has been applied to soybean to increase its stearic acid content (Kinney 1998a,b). It is interesting that, when soybean germplasm with increased stearic acid content was sexually crossed to the FAD2-1 downregulated high oleic acid germplasm, the resultant progeny produced an oil with stearic acid and oleic acid at 30% and 60%, respectively (Kinney 1996).

16:0-Δ4-ACP-desaturase

Cahoon and co-workers expressed a cDNA encoding a putative acyl-ACP desaturase from coriander into tobacco by *Agrobacterium tumifaciens*-mediated transformation (Cahoon et al. 1992). Accumulation of petroselinic acid (18:1-Δ6*cis*) and Δ4-hexadecenoic acid, both of which were absent from control callus, was observed. Later, these workers provided metabolic evidence for the involvement of this 16:0-Δ4-ACP-desaturase in

normal petroselinic acid synthesis by coriander endosperm and also by transgenic tobacco cells (Cahoon and Ohlrogge 1994).

A desaturase with 83% sequence identity to coriander 16:0-Δ4-ACP-desaturase was isolated from developing seeds of *Hedera helix* (English ivy) (Whittle et al. 2005). Expression of the ivy desaturase in *Arabidopsis* resulted in the accumulation of C16:1-Δ4 and its expected elongation product C18:1-Δ6 (petroselinic acid). In vitro desaturation reactions also revealed that 16:1-Δ9-ACP and C18:1-Δ9-ACP can be further metabolised to 16:2-Δ4,Δ9 and C18:2-Δ4,Δ9, respectively, by the ivy desaturase showing a capacity of this enzyme to perform two desaturations on the saturated and monounsaturated substrates. This is a new finding for the soluble class of desaturases (Whittle et al. 2005).

20:0-Δ5-desaturase

The cDNAs for enzymes involved in the biosynthesis of C20:1-Δ5 have been identified from developing *Limnanthes douglassii* seeds (Cahoon et al. 2000). Expression of a cDNA for the *L. douglassii* acyl-CoA desaturase homolog in somatic soybean embryos, behind a strong seed-specific promoter, resulted in the accumulation of Δ5-hexadecenoic acid in amounts of 2% to 3% (w/w) of the total fatty acids of single embryos. It is interesting to note that the authors also coexpressed cDNAs for *L. douglassii* acyl-CoA desaturase and FAE1 in order to partially reconstitute the biosynthetic pathway of C20:1-Δ5 in transgenic plant tissue. These transformations resulted in production of C20:1-Δ5 and Δ5-docosenoic acid which comprised up to 12% of the total fatty acids in transgenic embryos (Cahoon et al. 2000).

In the further study, a *L. douglassii* seed-specific cDNA (Lim Des5) encoding a homolog of acyl-CoA desaturase found in animals, fungi and cyanobacteria was expressed in *B. carinata*, which resulted in the accumulation of C22:2-Δ5,Δ13 acid (up to 10%) in the seed oil (Jadhav et al. 2005). In conclusion, these results demonstrated the potential utility of soybean and *B. carinata* for the production of vegetable oils containing novel C20 and C22 fatty acids, and confirmed the preferred substrates of the Lim Des5 as C20:0 and C22:1-Δ13 (Jadhav et al. 2005).

Expression of a Δ5-desaturase cDNA from a filamentous fungus *Mortierella alpina* in transgenic canola seeds resulted in the production of taxoleic acid (C18:2-Δ5,Δ9) and pinolenic acid (C18:3-Δ5,Δ9,Δ12) in amounts ranging from 0.9 to 6.2% of the total fatty acids in the seeds (Knutzon et al. 1998).

Δ6-desaturase

To enable the production of γ-linolenic acid (C18:3-Δ6,Δ9,Δ12; GLA) in conventional oilseeds, Sayanova and co-workers isolated a cDNA encoding the Δ6-fatty acid desaturase from developing seeds of borage (*Borago officinalis*) (Sayanova et al. 1997). Functional expression of this cDNA in transgenic tobacco plants showed the accumulation of GLA and octadecatetraenoic acid (C18:4-Δ6,Δ9,Δ12,Δ15) at levels of 13.2% and 9.6% of the total fatty acids, respectively. The borage Δ6-fatty acid desaturase differs from other desaturase enzymes by the presence of an N-terminal domain related to cytochrome b_5 (Sayanova et al. 1997). It is interesting that Δ6-unsaturated fatty acids were found in both plastidic and microsomal lipids and positional analysis revealed that these fatty acids accumulated predominantly at the *sn*-2 position of the glycerolipids unlike other unusual plant fatty acids (Sayanova et al. 1999). As the borage Δ6-desaturase is most probably located in the endoplasmic reticulum (ER), a possible import of Δ6-unsaturated fatty acids into plastids after desaturation in the ER was suggested (Sayanova et al. 1999).

Expression of a Δ6-desaturase from the oleaginous fungus, *Pythium irregulare*, in *Brassica juncea* (under the control of a napin promoter) resulted in production of three Δ6 unsaturated fatty acids (C18:2-Δ6,Δ9; C18:3-Δ6,Δ9,Δ12; and C18:4-Δ6,Δ9,Δ12,Δ15) in seeds (Hong et al. 2002). Among them, GLA was the most abundant and accounted for up to 40% of the total seed fatty acids. It has also been shown that GLA was incorporated into the TAG fraction (98.5%) with only trace amounts found in the other lipids (Hong et al. 2002). In another study, introduction of borage Δ6-desaturase into flax (*Linum usitatissimum*), under the control of a constitutive (35S) promoter, showed the accumulation of two Δ6-unsaturated fatty acids, C18:3-Δ6,Δ9,Δ12; and C18:4-Δ6,Δ9,Δ12,Δ15 (Qiu et al. 2002). The level of these fatty acids was up to 22% of the total fatty acids in the stem, 19% in the root and 11% in the leaf. Introduction of this desaturase in *B. juncea* under the control of a napin promoter resulted in synthesis of Δ6-fatty acids at levels of up to 13% of the total fatty acids in mature seeds (Qiu et al. 2002).

An increase in GLA and octadecatetraenoic acid in leaf tissues of a commercial variety of evening primrose (*Oenothera* sp.) has been achieved through a robust *Agrobacterium*-mediated transformation procedure to deliver a cDNA encoding a Δ6-desaturase from borage (De Gyves et al. 2004).

As an alternative to fish oil, oilseed plants have been also considered as a potential source of very-long chain polyunsaturated fatty acids (VLCPUFAs) if they are gene engineered (Alonso and Maroto 2002; Drexler et al. 2003; Abbadi et al. 2004). The accumulation of substantial amounts of

arachidonic and eicosapentaenoic acids has been achieved in *Arabidopsis thaliana* (Qi et al. 2004). This involved the use of genes encoding enzymes of the ω3/6-Δ8-desaturation biosynthetic pathways for the formation of C20 PUFAs. *A. thaliana* was transformed sequentially with genes encoding a Δ9-specific elongation activity from *Isochrysis galbana*, a Δ8-desaturase from *Euglena gracilis* and a Δ5-desaturase from *Mortierella alpina* (Qi et al. 2004). It was suggested that *I. galbana* C18-Δ9-elongation activity was important in the successful reconstitution of this pathway since it may bypass rate-limiting steps present in the conventional Δ6-desaturase/elongase pathway (Qi et al. 2004).

In transgenic tobacco and linseed, high accumulation of Δ6-desaturated C18 fatty acids and up to 5% of C20 PUFAs, including arachidonic and eicosapentaenoic acids, has been reported as a result of heterologous expression of three genes encoding a Δ6-desaturase, a Δ6-elongase and Δ5-desaturase (Abbadi et al. 2004).

An interesting aspect of the modulation of fatty acid desaturation has been suggested by Zhang and co-workers in causing an increased tolerance to various abiotic stresses in transgenic tobacco cells and plants (Zhang et al. 2005). They presented evidence that overexpression of either FAD3 or FAD8 led to increased tolerance to drought in tobacco plants and to osmotic stress in cultured cells. In both cultured cells and whole plants, much greater heat sensitivity was noted when the tissues were overexpressed with FAD8 rather than with FAD3 (Zhang et al. 2005).

4. Complex lipid manipulation

In spite of significant progress in genetic engineering of fatty acid synthesis in oilseeds to meet the increasing demand of industry, the high levels of these fatty acids which are necessary for industrial applications have not been achieved very often. The highest content of a potentially useful industrial oil (with the exception of lauric acid) accumulated in the seed oil of transgenic plants is 25% (Jaworski and Cahoon 2003). Taking into account a need for purification of the oils which involves some additional expense, levels of unusual fatty acids at up to 90-95% of total seed fatty acids have been suggested as desirable.

Analysis of the fractional distribution of unusual fatty acids in seed oils showed that they are located almost exclusively in TAGs. The main pathway for the synthesis of TAGs is the Kennedy pathway (Fig. 9.2) where the first two reactions are the formation of phosphatidic acid by stepwise acylation of glycerol 3-phosphate. It is known now that these reactions are

catalysed by two distinct enzymes specific for positions 1 and 2. Membrane-bound glycerol 3-phosphate acyltransferase (GPAT) initiates the process by transferring the acyl chain from CoA to the sn-1 position of glycerol-3-phosphate with the formation of lysophosphatidic acid (Voelker and Kinney 2001). No gene has been identified in plants for the membrane-bound form of GPAT, which is believed to have a low selectivity for different acyl chains. (The soluble chloroplast form of GPAT has, however, been well studied (Frentzen and Wolter 1998). The transfer of acyl chains from acyl-CoAs to the sn-2 position to form phosphatidic acid, is catalyzed by lysophosphatidic acid acyltransferase (LPAAT) which, in plants, prefers unsaturated acyl chains (Voelker and Kinney 2001). The phosphatidic acid is then dephosphorylated to produce diacylglycerol (DAG). The final step in the pathway is the addition of a final fatty-acyl group to the sn-3 position of DAG to produce TAG (Fig. 9.2). It is catalyzed by diacylglycerol acyltransferase (DGAT), an enzyme unique to TAG biosynthesis. In plants, two unrelated genes have been shown to potentially encode DGAT enzymes. One form is related to acyl-CoA:cholesterol acyltransferase, whereas a second form does not resemble any other known genes (see Voelker and Kinney 2001).

Recent studies provide evidence for alternative reactions for TAG synthesis in plants. In one of these reactions, a fatty acid residue is directly transferred from the sn-2 position of phosphatidylcholine (PC) to diacylglycerol forming lyso-PC and TAG (Stobart et al. 1997). There is also a reaction involving acyl transfer between two molecules of DAG (see Weselake 2005). Each of above-mentioned reactions in the TAG biosynthetic pathway may be a limiting step for the incorporation of fatty acids into TAG. Thus, in addition to the enzymes necessary for the synthesis of an unusual fatty acid, it may be necessary to introduce multiple genes to properly channel the fatty acids into TAG (Jaworski and Cahoon 2003).

In order to increase the erucic acid content in rapeseed oil, the cDNA encoding LPAAT from developing seeds of meadowfoam (*Limnanthes alba alba*) was expressed in developing seeds of transgenic high-erucic-acid rapeseed (Lassner et al. 1995). Although the total erucic acid content did not change, it was present at the sn-2 position of TAGs in transgenic plants in contrast to control plants, and trierucin was accumulated in the seed oil from modified plants (Lassner et al. 1995). Similar results were obtained when a cDNA encoding a 1-acyl-sn-3-phosphate acyltransferase (LPAAT) from *Limnanthes douglasii* was introduced into oilseed rape (Brough et al. 1996). Moreover, when a coconut (*Cocos nucifera*) LPAAT (preferring 12:0-CoA) was coexpressed with a 12:0-ACP thioesterase from California bay in developing seeds of oilseed rape, efficient laurate deposition

at the sn-2 position of TAGs and trilaurin accumulation were found (Knutzon et al. 1999).

Zou et al. (1997) transformed the model oilseed arabidopsis and a high-erucic acid cultivar of *Brassica napus* with the yeast sn-2 acyltransferase gene. They reported substantial increases in seed oil content in transgenic plants as well as increases in both the overall proportions and the amounts of VLCFAs in seed TAGs. The proportion of these acids at the sn-2 position of TAGs was also increased in transgenic plants (Zou et al. 1997). Enhancement of seed oil content has been achieved in *A. thaliana* when transformed with a plastidial safflower glycerol 3-phosphate acyltransferase (GPAT) and an *Escherichia coli* GPAT (Jain et al. 2000).

The potential application of acyl-CoA:diacylglycerol acyltransferase-transformed plants has been demonstrated when transformation of tobacco with the DGAT gene isolated from *A. thaliana* was performed (Bouvier-Nave et al. 2000). In several primary transformants, a marked increase of TAG content, which correlated with the DGAT mRNA expression, has been observed (Bouvier-Nave et al. 2000). Moreover, it was shown that seed-specific overexpression of the DGAT cDNA in wild-type arabidopsis enhanced oil deposition and average seed weight which were correlated with DGAT transcript levels (Jako et al. 2001). This study confirmed the important role of DGAT in regulating the quantity of seed TAGs (Jako et al. 2001).

5. Summary remarks

While there is clearly enormous potential for the production of important foodstuffs and renewable chemical sources by use of genetic manipulation of plants, there has been little realisation of this potential so far. A notable exception is laurate canola. Two major problems have been encountered. First, achievement of high levels of the desired fatty acids in TAG has often been limited by the substrate selectivity of endogenous enzymes. This means that, frequently, it appears necessary to introduce novel acyltransferases in order to reduce constraints encountered in the Kennedy pathway. Second, when trying to produce products such as VLCPUFAs, there is an inherent problem in that the desaturation and elongation reactions use different types of substrates and it is constantly necessary to swap the acyl groups between these during synthesis. Such additional reactions severely limit the efficiency of the syntheses achieved so far.

There are two further problems. One of these is our lack of information about control mechanisms for lipid synthesis in different plants (Ohlrogge

and Jaworski 1997). In the first attempts to address this deficiency, flux control analysis is being used to yield quantitative information about the regulation of lipid accumulation (e.g. Ramli et al. 2002; 2005). Such experiments are very necessary in order to identify which enzymes may be worth manipulating in order to achieve increased oil yields.

A second problem is that of consumer resistance to the use of genetically-modified crops. At present such problems are more serious in Europe then many other parts of the World. Nevertheless, they have had a major effect on the development of new crops. However, for the production of new crops for industrial use there is somewhat less public disquiet and it is probable that advances in the production of renewable resources for chemicals are likely to be very significant in the near future.

Abbreviations used

ACC, acetyl-CoA carboxylase
ACP, acyl carrier protein
BTE, fatty acyl thioesterase type B (FatB c.f. FatA)
DAG, diacylglycerol
DGAT, diacylglycerol acyltransferase
E, fatty acid elongase
FAD, fatty acid desaturase
FAE, fatty acid elongase
FAR, fatty acyl-CoA reductase
FAS, fatty acid synthase
GLA, gamma-linolenic acid
GPAT, glycerol 3-phosphate acyltransferase
HEAR, high erucic acid rape
KAS, β-ketoacyl-ACP synthase
KCS, β-ketoacyl-CoA synthase
LPAAT, lysophosphatidic acid acyltransferase
PC, phosphatidylcholine
PUFA, polyunsaturated fatty acid
TAG, triacylglycerol
VLCFA, very long chain (>18C) fatty acid

References

Abbadi A, Domergue F, Bauer J, Napier JA, Welti R, Zähringer U, Cirpus P, Heinz E (2004) Biosynthesis of very-long-chain polyunsaturated fatty acids in transgenic oilseeds: constraints on their accumulation. Plant Cell 16: 2734-2748

Alonso DL, Maroto FG (2000) Plants as "chemical factories" for the production of polyunsaturated fatty acids. Biotechnol Adv 18: 481-497

Bafor M, Smith MA, Jonsson L, Stobart K, Stymne S (1993) Biosynthesis of vernoleate (cis-12-epoxyoctadeca-cis-9-enoate) in microsomal preparations from developing endosperm of *Euphorbia lagascae*. Arch Biochem Biophys 303: 145-151

Bouvier-Nave P, Benveniste P, Oelkers P, Sturley SL, Schaller H (2000) Expression in yeast and tobacco of plant cDNA encoding acyl-CoA:diacylglycerol acyltransferase. Eur J Biochem 267: 85-96

Broadwater JA, Whittle E, Shanklin J (2002) Desaturation and hydroxylation. Residues 148 and 324 of *Arabidopsis* FAD2, in addition to substrate chain length, exert a major influence in partitioning of catalytic specificity. J Biol Chem 277: 15613-15620

Brough CL, Coventry JM, Christie WW, Kroon JTM, Brown AP, Barsby TL, Slabas AR (1996) Towards genetic engineering of triacylglycerols of defined fatty acid composition: major changes in erucic acid content at the sn-2 position affected by the introduction of a 1-acyl-sn-glycerol-3-phosphate acyltransferase from *Limnanthes douglasii* into oil seed rape. Mol Breeding 2: 133-142

Broun P, Somerville C (1997) Accumulation of ricinoleic, lesquerolic, and densipolic acids in seeds of transgenic *Arabidopsis* plants that express a fatty acyl hydroxylase cDNA from castor bean. Plant Physiol 113: 933-942

Broun P, Boddupalli S, Somerville C (1998a) A bifunctional oleate 12-hydroxylase: desaturase from *Lesquerella fendleri*. Plant J 13: 201-210

Broun P, Shanklin J, Whittle E, Somerville C (1998b) Catalytic plasticity of fatty acid modification enzymes underlying chemical diversity of plant lipids. Science 282: 1315-1317

Cahoon EB, Shanklin J, Ohlrogge JB (1992) Expression of a coriander desaturase results in petroselinic acid production in transgenic tobacco. Proc Natl Acad Sci USA 89: 11184-11188

Cahoon EB, Ripp KG, Hall SE, Kinney AJ (2001) Formation of conjugated delta(8), delta(10)-double bonds by delta(12)-oleic-acid desaturase-related enzymes – biosynthetic origin of calendic acid. J Biol Chem 276: 2637-2643

Cahoon EB, Carlson TJ, Ripp KG, Schweiger BJ, Cook GA, Hall SE, Kinney AJ (1999) Biosynthetic origin of conjugated double bonds: production of fatty acid components of high-value drying oils in transgenic soybean embryos. Proc Natl Acad Sci USA 96: 12935-12940

Cahoon EB, Ohlrogge JB (1994) Metabolic evidence for the involvement of a delta(4)-palmitoyl-acyl carrier protein desaturase in petroselinic acid synthesis

in coriander endosperm and transgenic tobacco cells. Plant Physiol 104: 827-837

Cahoon EB, Marillia EF, Stecca KL, Hall SE, Taylor DC, Kinney AJ (2000) Production of fatty acid components of meadowfoam oil in somatic soybean embryos. Plant Physiol 124: 243-251

Cahoon EB, Ripp KG, Hall SE, McGonigle B (2002) Transgenic production of epoxy fatty acids by expression of a cytochrome P450 enzyme from *Euphorbia lagascae* seed. Plant Physiol 128: 615-624

Cahoon EB, Kinney AJ (2004) Dimorphecolic acid is synthesized by the coordinate activities of two divergent Δ^{12}-oleic acid desaturases. J Biol Chem 279: 12495-12502

Chapman KD, Austin-Brown S, Sparace SA, Kinney AJ, Ripp KG, Pirtle IL, Pirtle RM (2001) Transgenic cotton plants with increased seed oleic acid content. J Amer Oil Chem Soc 78: 941-947

Davies HM (1996) Engineering new oilseed crops from rapeseed. In: Janick J (ed) Progress in new crops. ASHS Press, Alexandria, VA, pp 299-306

De Gyves EM, Sparks CA, Sayanova O, Lazzeri P, Napier JA, Jones HD (2004) Genetic manipulation of gamma-linolenic acid (GLA) synthesis in a commercial variety of evening primrose (*Oenothera* sp.). Plant Biotech J 2: 351-357

Dehesh K, Jones A, Knutzon DS, Voelker TA (1996) Production of high levels of 8:0 and 10:0 fatty acids in transgenic canola by overexpression of Ch FatB2, a thioesterase cDNA from *Cuphea hookeriana*. Plant J 9: 167-172

Dehesh K, Tai H, Edwards P, Byrne J, Jaworski JG (2001) Overexpression of 3-ketoacyl-acyl carrier protein synthase III in plants reduced the rate of lipid synthesis. Plant Physiol 125: 1103-1114

Dörmann P (2005) Membrane lipids. In: Murphy DJ (ed) Plant Lipids: Biology, utilisation and manipulation. Blackwell, Oxford, pp 123-161

Drexler H, Spiekermann P, Meyer A, Domergue F, Zank T, Sperling P, Abbadi A, Heinz E (2003) Metabolic engineering of fatty acids for breeding of new oilseed crops: strategies, problems and first results. J Plant Physiol 160: 779-802

Dyer JM, Chapital DC, Kuan JC, Mullen RT, Turner C, McKeon TA, Pepperman AB (2002) Molecular analysis of a bifunctional fatty acid conjugase/desaturase from tung. Implications for the evolution of plant fatty acid diversity. Plant Physiol 130: 2027-2038

Eccleston VS, Cranmer AM, Voelker TA, Ohlrogge JB (1996) Medium-chain fatty acid biosynthesis and utilization in *Brassica napus* plants expressing lauroyl-acyl carrier protein thioesterase. Planta 198: 46-53

Eccleston VS, Ohlrogge JB (1998) Expression of lauroyl-acyl carrier protein thioesterase in *Brassica napus* seeds induces pathway for both fatty acid oxidation and biosynthesis and implies a set point for triacylglycerol accumulation. Plant Cell 10: 613-622

Frentzen M, Wolter FP (1998) Molecular biology of acyltransferases involved in glycerolipid synthesis. In: Harwood JL (ed) Plant lipid biosynthesis: fundamentals and agricultural applications. Cambridge University Press, New York, pp 247-272

Gurr MI, Harwood JL, Frayn KN (2002) Lipid biochemistry. An introduction. 5th edt. Blackwell, Oxford

Harwood JL (1996) Recent advances in the biosynthesis of plant fatty acids. Biochim Biophys Acta 1301: 7-56

Harwood JL (2005) Fatty acid biosynthesis. In: Murphy DJ (ed) Plant lipids: biology, utilisation and manipulation. Blackwell, Oxford, pp 27-66

Hatanaka T, Shimizu R, Hildebrand D (2004) Expression of a *Stokesia laevis* epoxygenase gene. Phytochem 65: 2189-2196

Hawkins DJ, Kridl JC (1998) Characterization of acyl-ACP thioesterases of mangosteen (*Garcinia mangostana*) seed and high levels of stearate production in transgenic canola. Plant J 13: 743-752

Hildebrand DF, Yu K, McCracken C, Rao SS (2005) Fatty acid manipulation. In: Murphy DJ (ed) Plant lipids: biology, utilisation and manipulation. Blackwell, Oxford, pp 67-102

Hong H, Datla N, Reed DW, Covello PS, MacKenzie SL, Qiu X (2002) High-level production of γ-linolenic acid in *Brassica juncea* using a Δ6 desaturase from *Pythium irregulare*. Plant Physiol 129: 354-362

Hornung E, Pernstich C, Feussner I (2002) Formation of conjugated delta11,delta13-double bonds by delta12-linoleic acid (1,4)-acyl-lipid-desaturase in pomegranate seeds. Eur J Biochem 269: 4852-4859

Hornung E, Krueger C, Pernstich C, Gipmans M, Porzel A, Feussner I (2005) Production of (10E, 12Z)-conjugated linoleic acid in yeast and tobacco seeds. Biochim Biophys Acta 30: 105-114

Iwabuchi M, Kohno-Murase J, Imamura J (2003) Delta 12-oleate desaturase-related enzymes associated with formation of conjugated trans-delta 11, cis-delta 13 double bonds. J Biol Chem 278: 4603-4610

Jadhav A, Marillia EF, Babic V, Giblin EM, Cahoon EB, Kinney AJ, Mietkiewska E, Brost JM, Taylor DC (2005) Production of 22:2(delta 5, delta 13) and 20:1 (delta 5) in *Brassica carinata* and soybean breeding lines via introduction of *Limnanthes* genes. Mol Breed 15: 157-167

Jain RK, Coffey M, Lai K, Kumar A, MacKenzie SL (2000) Enhancement of seed oil content by expression of glycerol-3-phosphate acyltransferase genes. Biochem Soc Trans 28: 958-961

Jako C, Kumar A, Wei Y, Zou J, Barton DL, Giblin EM, Covello PS, Taylor DC (2001) Seed-specific over-expression of an *Arabidopsis* cDNA encoding a diacylglycerol acyltransferase enhances seed oil content and seed weight. Plant Physiol 126: 861-874

Jaworski J, Cahoon EB (2003) Industrial oils from transgenic plants. Curr Opin Plant Biol 6: 178-184

Kinney AJ (1996) Development of genetically engineered soybean oils for food application. J Food Lipids 3: 273-292

Kinney AJ (1998a) Plants as industrial chemical factories – new oils from genetically engineered soybeans. Fett Lipid 100: 173-179

Kinney AJ (1998b) Manipulating flux through plant metabolic pathways. Curr Opin Plant Biol 1: 173-178

Kinney AJ, Clemente TE (2005) Modifying soybean oil for enhanced performance in biodiesel blends. Fuel Process Technol 86: 1137-1147

Knutzon DS, Thompson GA, Radke SE, Johnson WB, Knauf VC, Kridl JC (1992) Modification of *Brassica* seed oil by antisense expression of a stearoyl-acyl carrier protein desaturase gene. Proc Natl Acad Sci USA 89: 2624-2628

Knutzon DS, Thurmond JM, Huang YS, Chaudhary S, Bobik EG, Chan GM, Kirchnert SJ, Mukerji P (1998) Identification of Δ5-desaturase from *Mortierella alpina* by heterologous expression in bakers' yeast and canola. J Biol Chem 273: 29360-29366

Knutzon DS, Hayes TR, Wyrick A, Xiong H, Davies HM, Voelker TA (1999) Lysophosphatidic acid acyltransferase from coconut endosperm mediates the insertion of laurate at the sn-2 position of triacylglycerols in lauric rapeseed oil and can increase total laurate levels. Plant Physiol 120: 739-746

Kohno-Murase J, Iwabuchi M, Endo-Kasahara S, Sugita K, Ebinuma H, Imamura J (2006) Production of trans-10, cis-12 conjugated linoleic acid in rice. Transgenic Res 15: 95-100

Kridl JC (1998) Engineering canola vegetable oil for food and industrial uses. In: Shewry PR, Napier JA, Davis PJ (eds) Engineering crop plants for industrial end uses. Portland, London, pp 159-169

Lardizabal KD, Metz JG, Sakamoto T, Hutton WC, Pollard MR, Lassner MW (2000) Purification of a jojoba embryo wax synthase, cloning of its cDNA, and production of high levels of wax in seeds of transgenic *Arabidopsis.* Plant Physiol 122: 645-655

Lassner MW, Levering CK, Davies HM, Knutzon DS (1995) Lysophosphatidic acid acyltransferase from meadowfoam mediates insertion of erucic acid at the sn-2 position of triacylglycerol in transgenic rapeseed oil. Plant Physiol 109: 1389-1394

Lassner MW, Lardizabal K, Metz JC (1996) A jojoba beta-ketoacyl-CoA synthase cDNA complements the canola fatty acid elongation mutation in transgenic plants. Plant Cell 8: 281-292

Lee M, Lenman M, Banas A, Bafor M, Singh S, Schweizer M, Nilsson R, Liljenberg C, Dahlqvist A, Gummeson PO, Sjödahl S, Green A, Stymne S (1998) Identification of non-heme diiron proteins that catalyze triple bond and epoxy group formation. Science 280: 915-918

Leonard JM, Knapp SJ, Slabaugh MB (1998) A *Cuphea* beta-ketoacyl-ACP synthase shifts the synthesis of fatty acids towards shorter chains in *Arabidopsis* seeds expressing *Cuphea* FatB thioesterases. Plant J 13: 621-628

Liu Q, Singh S, Green A (2000) Genetic modification of cotton seed oil using inverted-repeat gene-silencing technique. Biochem Soc Trans 28: 927-929

Mekhedov S, de Ilarduya OM, Ohlrogge JB (2000) Toward a functional catalog of the plant genome. A survey of genes for lipid biosynthesis. Plant Physiol 122: 389-401

Metz JG, Pollard MR, Anderson L, Hayes TR, Lassner MW (2000) Purification of a jojoba embryo fatty acyl-coenzyme A reductase and expression of its cDNA in high erucic acid rapeseed. Plant Physiol 122: 635-644

Mietkiewska E, Giblin EM, Wang S, Barton DL, Dirpaul J, Brost JM, Katavic V, Taylor DC (2004) Seed-specific heterologous expression of a nasturtium FAE gene in *Arabidopsis* results in a dramatic increase in the proportion of erucic acid. Plant Physiol 136: 2665-2675

Moon H, Smith MA, Kunst L (2001) A condensing enzyme from the seeds of *Lesquerella fendleri* that specifically elongates hydroxyl fatty acids. Plant Physiol 127: 1635-1643

Murphy DJ (2005) The study and utilisation of plant lipids: from margarine to lipid rafts. In: Murphy DJ (ed) Plant lipids: biology, utilisation and manipulation. Blackwell, Oxford, pp 1-26

O'Hara P, Slabas AR, Fawcett T (2000) Modulation of fatty acid biosynthesis by β-keto reductase expression. Biochem Soc Trans 28: 613-615

Ohlrogge JB, Jaworski JG (1997) Regulation of fatty acid synthesis. Annu Rev Plant Physiol Plant Mol Biol 48: 109-136

Qi BX, Fraser T, Mugford S, Dobson G, Sayanova O, Butler J, Napier JA, Stobart AK, Lazarus CM (2004) Production of very long chain polyunsaturated omega-3 and omega-6 fatty acids in plants. Nat Biotechnol 22: 739-745

Qiu X, Hong HP, Datla N, MacKenzie SL, Taylor DC, Thomas TL (2002) Expression of borage delta 6 desaturase in *Saccharomyces cerevisiae* and oilseed crops. Can J Bot 80: 42-49

Ramli US, Baker DS, Quant PA, Harwood JL (2002) Control analysis of lipid biosynthesis in tissue cultures from oil crops shows that flux control is shared between fatty acid synthesis and lipid assembly. Biochem J 364: 393-401

Ramli US, Salas JJ, Quant PA, Harwood JL (2005) Metabolic control analysis reveals an important role for diacylglycerol acytransferase in olive but not in oil palm lipid accumulation. FEBS J 272: 5764-5770

Sayanova O, Davies GM, Smith MA, Griffiths G, Stobart AK, Shewry PR, Napier JA (1999) Accumulation of delta(6)-unsaturated fatty acids in transgenic tobacco plants expressing a delta(6)-desaturase from *Borago officinalis.* J Exptl Bot 50: 1647-1652

Sayanova O, Smith MA, Lapinskas P, Stobart AK, Dobson G, Christie WW, Shewry PR, Napier JA (1997) Expression of a borage desaturase cDNA containing an N-terminal cytochrome b_5 domain results in the accumulation of high levels of Δ^6 –desaturated fatty acids in transgenic tobacco. Proc Natl Acad Sci USA 94: 4211-4216

Singh S, Thomaeus S, Lee M, Stymne S, Green A (2001) Transgenic expression of a delta 12-epoxygenase gene in *Arabidopsis* seeds inhibits accumulation of linoleic acid. Planta 212: 872-879

Slabas AR, Sanda SL (1998) Complex lipid biosynthesis and its manipulation in plants. In: Shewry PR, Napier JA, Davis PJ (eds) Engineering crop plants for industrial end uses. Portland, London, pp 171-179

Slabas AR, Chase D, Nishida I, Murata N, Sidebottom C, Safford R, Sheldon PS, Kekwick RGO, Hardie DG, MacKintosh RW (1992) Molecular-cloning of higher-plant 3-oxoacyl-(acyl carrier protein) reductase – sequence identities with the nodG-gene product of the nitrogen-fixing soil bacterium *Rhizobium meliloti*. Biochem J 283: 321-326

Smith M, Moon H, Kunst L (2000) Production of hydroxyl fatty acids in the seeds of *Arabidopsis thaliana*. Biochem Soc Trans 28: 947-950

Stobart K, Mancha M, Lenman M, Dahlqvist A, Stymne S (1997) Triacylglycerols are synthesized and utilized by transacylation reactions in microsomal preparations of developing safflower (*Carthamus tinctorius* L.) seeds. Planta 203: 58-66

Stoutjesdijk PA, Hurlestone C, Singh SP, Green AG (2000) High-oleic acid Australian *Brassica napus* and *B. juncea* varieties produced by co-supression of endogenous Δ12-desaturase. Biochem Soc Trans 28: 938-940

Van de Loo FJ, Broun P, Turner S, Somerville C (1995) An oleate 12-hydroxylase from *Ricinus communis* L. is a fatty acyl desaturase homolog. Proc Natl Acad Sci USA 92: 6743-6447

Verwoert IIGS, van der Linden KH, Walsh MC, Nijkamp HJJ, Stuitje AR (1995) Modification of *Brassica napus* seed oil by expression of the *Escherichia coli* fab H gene, encoding 3-ketoacyl-acyl carrier protein synthase III. Plant Mol Biol 27: 875-886

Voelker TA, Worrell AC, Anderson L, Bleibaum J, Fan C, Hawkins DJ, Radke SE, Davies HM (1992) Fatty acid biosynthesis redirected to medium chains in transgenic oilseed plants. Science 257: 72-74

Voelker T, Kinney AJ (2001) Variations in the biosynthesis of seed-storage lipids. Annu Rev Plant Physiol Plant Mol Biol 52: 335-361

Weselake RJ (2005) Storage lipids. In: Murphy DJ (ed) Plant lipids: biology, utilisation and manipulation. Blackwell, Oxford, pp 162-225

Whittle E, Cahoon EB, Subrahmanyam S, Shanklin J (2005) A multifunctional acyl-acyl carrier protein desaturase from *Hedera helix* L. (English ivy) can synthesize 16- and 18-carbon monoene and diene products. J Biol Chem 280: 28169-28176

Yuan L, Voelker TA, Hawkins DJ (1995) Modification of the substrate specificity of an acyl-acyl carrier protein thioesterase by protein engineering. Proc Natl Acad Sci USA 92: 10639-10643

Zhang M, Barg R, Yin M, Gueta-Dahan Y, Leikin-Frenkel A, Salts Y, Shabtai S, Ben-Hayyim G (2005) Modulation of fatty acid desaturation via overexpression of two distinct omega-3 desaturases differentially alters tolerance to various abiotic stresses in transgenic tobacco cells and plants. Plant J 44: 361-371

Zou J, Katavic V, Giblin EM, Barton DL, MacKenzie SL, Keller WA, Hu X, Taylor DC (1997) Modification of seed oil content and acyl composition in the *Brassicaceae* by expression of a yeast sn-2 acyltransferase gene. Plant Cell 9: 909-923

10

Biodiesel production

Jon Van Gerpen

Department of Biological and Agricultural Engineering, 419 Engineering Physics Building, University of Idaho, P.O. Box 440904, Moscow (e-mail: jonvg@uidaho.edu)

1. Introduction

Biodiesel is a fuel for diesel engines that consists of the mono-alkyl esters of fatty acids from vegetable oils and animal fats. It can be used either pure, or in blends with petroleum-based diesel fuel. It does not require that engines or fueling infrastructure be modified although checking elastomers for compatibility is recommended when pure biodiesel (B100) is used. Biodiesel allows diesel engines to produce lower exhaust emissions of smoke, particulate, carbon monoxide, and unburned hydrocarbons. Oxides of nitrogen may increase slightly under some operating conditions but the difference for biodiesel blends up to 20% is difficult to measure.

Biodiesel provides a major new market for excess supplies of vegetable oil and animal fats. The reduction in these excess supplies should improve prices for commodity oilseeds such as soybeans and canola. It could also encourage the development of greater diversity in crop production as oilseeds with greater oil yield per acre become economically viable.

Biodiesel can also displace a portion of the petroleum used for transportation and, because it is biologically-based, may reduce the impact of global warming. However, its impact on these issues should not be exaggerated. Current world production of vegetable oils and animal fats and competition for these oils and fats from food uses will limit biodiesel's contribution to less than 5% of petroleum diesel fuel for the foreseeable

P. Ranalli (ed.), Improvement of Crop Plants for Industrial End Uses, 281–289.

future. So, while biodiesel's impact on these problems is positive, it will be small.

Biodiesel provides an improvement in diesel fuel lubricity, even when used in blends as low as 1 to 2%. This benefit is increasingly important as low-lubricity desulfurized diesel fuels are required. Recent work suggests that the actual lubricity benefit may be associated with contaminants in the biodiesel, such as free fatty acids and monoglycerides, rather than the mono-alkyl esters themselves (Knothe G. and Steidley KR 2005).

The combination of high petroleum prices and government incentives has caused a surge in interest in biodiesel in the United States. Production capacity is increasing rapidly. This paper describes the processing technology that is being used to produce the fuel in the United States.

2. Oil preparation

Figure 10.1 shows a schematic diagram of the process to convert oilseeds to biodiesel. Oil is extracted from vegetable sources using hexane as a solvent, or with a mechanical screw press.

A by-product of the oil extraction is meal, which in the case of soybeans and canola, is a high protein animal feed. For soybeans, the meal has higher value than the oil. The crude oil contains contaminants such as phospholipids, free fatty acids, and water that should be removed before converting the oil to biodiesel. The phospholipids, or gums, are removed by adding water to the oil while heating so the gums react with the water to form an insoluble sediment that can be removed by centrifugation, filtration, or settling and sold as crude lecithin. A fraction of the gums may require the addition of phosphoric acid to complete the hydration process so that all of the gums can be removed. Solvent-extracted soybean oil typically contains gum compounds equivalent to 900 ppm of phosphorus. Levels below 50 ppm are recommended for biodiesel production (Van Gerpen and Dvorak 2002).

To remove free fatty acids from the oil, a solution of sodium hydroxide in water is added to convert the free fatty acids to soap. Then, the soap is washed from the oil with water and the oil is dried. The resulting material, known as soapstock, contains the soap, unseparated oil, and water.

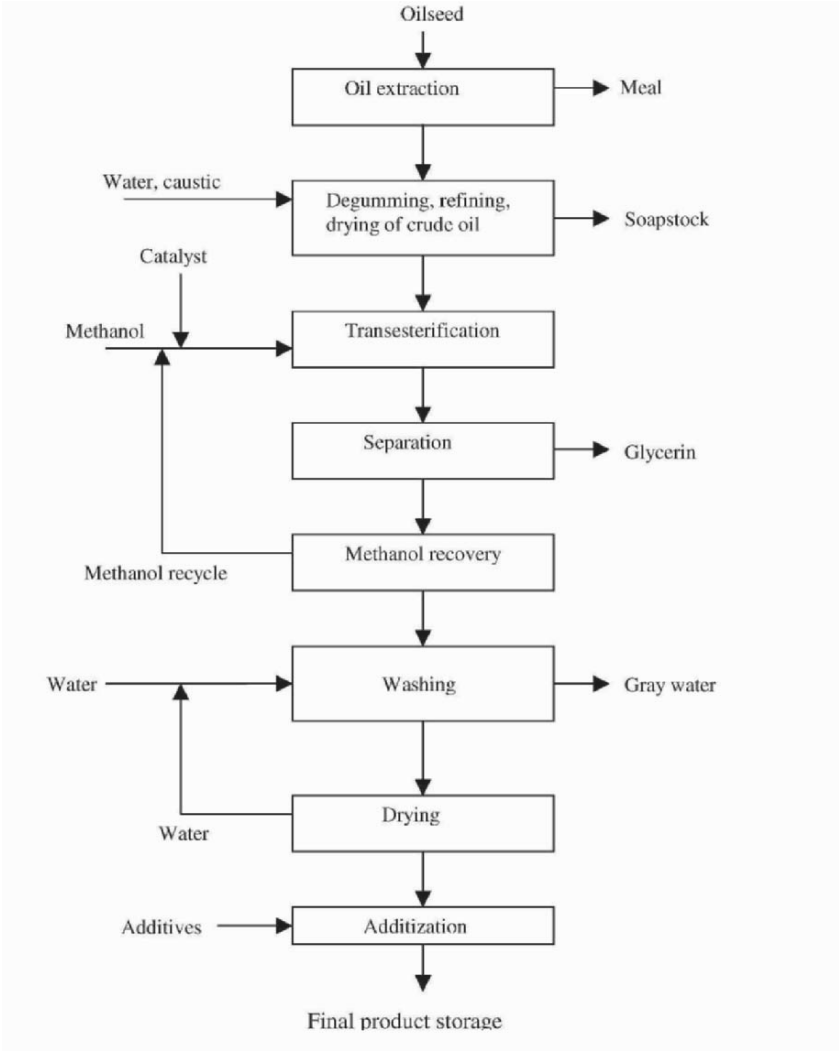


Fig. 10.1. Schematic diagram of Biodiesel production process.

The by-products of oil refining, lecithin and soapstock, both have uses in the production of human and animal food products. In addition to the refining processes described above, oil that is being prepared for human consumption is generally bleached and deodorized (Erickson 1995; Zehnder 1995). However, these steps are unnecessary for biodiesel production.

Some oils and fats contain high levels of free fatty acids. If these were processed using the caustic stripping process described above, the large amounts of soapstock would be a significant loss of useful product. The difficulty with leaving the free fatty acids in the oil for biodiesel production is that when the oil is subjected to the alkali-catalyzed transesterification process described below, the catalyst reacts quickly with the free fatty acids to form soap. This removes the catalyst from the reaction and the soap inhibits the biodiesel-glycerin separation process.

If the level of free fatty acids is less than about 5%, the oil or fat may still be processed with an alkali catalyst. In this case, an amount of extra catalyst is added to compensate for that lost to reaction with the free fatty acids and which ensures sufficient catalyst will remain to catalyze the reaction. Above 5%, the amount of soap inhibits separation of the glycerin.

When the free fatty acid level is above 5%, the excessive soap formation and the reduction in yield makes it necessary to use other means to process the feedstock. The most common method is to add a significant excess of methanol (commonly a 20:1 molar ratio of methanol to free fatty acids) with a strong acid, such as sulfuric acid (Canakci and Van Gerpen 2001). The acid catalyzes the esterification reaction of the fatty acids to methyl esters. When the level of free fatty acids has been reduced below 0.5%, the mixture can be passed through the transesterification process as if it were a refined oil. No soap is formed in the esterification reaction, but water is produced that must be removed before the mixture is transesterified or soap formation will be excessive.

3. Transesterification and separation

Transesterification is the process that takes a triglyceride molecule, itself an ester, and reacts it with an alcohol such as methanol to form the monoesters (biodiesel) and another alcohol (glycerol). This is an equilibrium reaction that stops well short of completion when the stoichiometric amount of methanol is added to the triglycerides. To force the equilibrium to the point where more than 98% of the glycerin has been separated from the triglycerides to produce biodiesel, at least 100% excess alcohol is required (Freedman and Pryde 1982). This degree of reaction is necessary to meet the biodiesel specification established by the American Society for Testing

and Materials (ASTM). Incomplete reaction leaves excessive amounts of monoglycerides in the biodiesel. These partially reacted products have a low solubility in the biodiesel and the saturated monoglycerides will form crystals that appear as white flakes in the fuel. Once formed, the crystals can be difficult to re-dissolve, particularly after blending with diesel fuel. If present in sufficient quantity, these crystals can plug fuel filters.

The most common alcohol used for transesterification is methanol, but ethanol can and has been used (Peterson et al. 1991). Ethanol requires more alcohol to be used and is usually more expensive. However, the extra weight of ethanol is converted to biodiesel so the higher cost of the ethanol may be offset by the greater yield.

Both hydroxide and methoxide catalysts are commonly used to produce biodiesel. Sodium or potassium hydroxide are dissolved in methanol to produce methoxide ions, which are the actual catalytic agents. Unfortunately, hydroxide catalysts also produce water which results in hydroxyl ions. These hydroxyls provide a parallel reaction path that produces free fatty acids and soaps instead of the methyl esters which are sought. Most commercial biodiesel producers in the United States use sodium methoxide which is formed via a water-free process and is available in concentrated solutions of 25% or 30% in methanol. Solid-phase, or heterogeneous, catalysts are being developed for biodiesel production and will probably see widespread use in the next 2 to 3 years.

The transesterification process can be conducted in a batch reactor where a typical reaction time is one hour at 60ºC. Continuously stirred tank reactors (CSTRs) are most commonly used in continuous plants. These operations usually use two CSTRs in series with approximately an hour of average residence time in each reactor. Glycerin separation between the two CSTRs helps to force the reaction equilibrium toward complete conversion of the triglycerides to methyl esters. The first reactor provides 75-90% of the reaction and the second reactor completes the process. Plug flow reactors can also be used but are less common due to the difficulty of providing sufficient reaction time.

Glycerin separation can be accomplished by gravity with a decanter, with a centrifuge, or with coalescence technology. The glycerin produced will only be about 50% purity having attracted about half of the excess methanol and most of the catalyst and soaps. Refining of glycerin to the 99.5+% level required for most commercial glycerin applications is generally not done by biodiesel producers. It is more common to upgrade the glycerin to 80% purity, which will be accepted by commercial glycerin refiners. This upgrading is accomplished by acidulating the glycerin with a strong acid such as hydrochloric, sulfuric or phosphoric, to neutralize the catalyst and split the soaps. Since the free fatty acids produced by the soap splitting are not soluble in the glycerin, they can be removed and used for

biodiesel production or introduced into animal feed. Then, the methanol is recovered from the glycerin by evaporation. This methanol may contain substantial amounts of water that must be removed, usually by fractional distillation, before the methanol can be returned to the reaction. After these processes, the glycerin will be 80 to 90% pure, depending on the contaminants in the original oil.

4. Methanol recovery and washing

After the biodiesel has been separated from the glycerin, it will contain small amounts of residual catalyst, soap, free glycerin and approximately 3 to 5% methanol. Excessive catalyst and soap will cause ash deposits in the engine and are controlled by the sulfated ash limitation in the ASTM specification. Free glycerin can cause filter plugging, injection system damage, and engine shutdown. Residual methanol causes a low flash point temperature and makes the fuel less safe to handle.

These contaminants must be removed before the fuel will meet the ASTM specification used in the United States. There are two general approaches in common use to remove these impurities. In the approach depicted in Figure 10.1, the methanol is removed first by evaporation with either a flash or falling film evaporator. Then, the methanol-free biodiesel is washed with water. The water might be sprayed into the biodiesel, mixed with the biodiesel via bubble-induced agitation, or contacted in counterflow in a packed or agitated extraction column. Other variations involve adding acids such as citric or hydrochloric to the initial wash water to neutralize the catalyst and possibly split the soaps into free fatty acids and salts. The free fatty acids will remain in the fuel and the salts are easily removed with subsequent water washing. A flash drying process follows the last contact with water to ensure that residual water levels in the fuel are low. If the biodiesel is not dried in this manner it may appear cloudy due to the presence of small water droplets suspended in the fuel. In this case, it will generally become clear with time as the droplets settle to the bottom of the holding tank.

An alternative process is to add wash water to the biodiesel immediately after the glycerin separation as shown in Figure 10.2. As before, acid is often added to the initial wash water for neutralization and soap splitting. Along with the soaps, salts, and free glycerin, most of the methanol will be transferred from the biodiesel to the wash water. The biodiesel is passed through a final flash vaporization process to ensure that any remaining methanol and water are removed.

The first process, where methanol removal precedes water washing, has the advantage of providing methanol with a low water concentration. Since most of the water that may have entered the reaction as a contaminant in the oil or methanol will have been extracted with the glycerin after the first stage of reaction, the methanol removed from the biodiesel probably has a low enough water content to be returned to the reaction with no further processing. In contrast, the methanol recovered from the biodiesel with the second process will contain 50 to 90% water and requires separation by fractional distillation. However, the first process has the disadvantage that when the methanol is vaporized, if the soap concentration is too high, it will precipitate out as a viscous sludge. When present, the methanol acts as a co-solvent and holds the soap in solution. This precipitated soap can plug filters and screens and complicate the washing process.

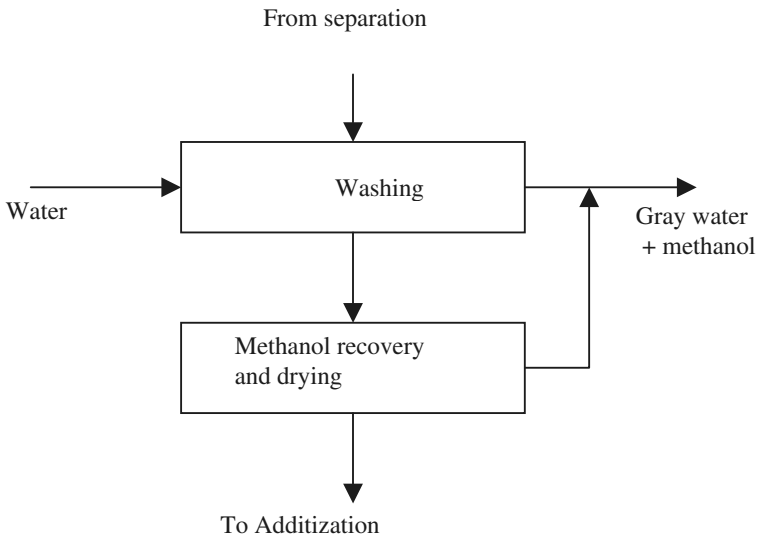


Fig. 10.2. Alternative washing process.

The acidulation that starts the second process breaks up the soap and thus eliminates this precipitation problem. The second process also has the advantage that only one vaporization process is required (because the water/methanol separation as been deferred to the distillation column) while the first process requires separate methanol and water removal processes. The first process is probably more appropriate when processing clean refined oils with low water concentrations that will produce little soap. The

second process is more robust for processes where higher soap levels are expected.

With either process, gray water is produced. In addition to soaps, the water may contain methanol, free glycerin, salts, and catalyst. The water will have a high biological oxygen demand and would require neutralization and dilution before being sent to a waste water treatment plant. A more common option is to add the gray water stream to the crude glycerin. The acidulation of the glycerin will also split the soaps in the water and the water will be removed by the vaporization process that recovers the methanol. After distillation to separate the methanol and water, the methanol can be reused for the transesterification process and the water can be reused for washing. The salts remain in the glycerin.

Alternatives to water washing are being proposed. Most involve adsorbents such as magnesium silicate or ion exchange resins. These materials are effective at removing soap, catalyst, free glycerin and, in some cases, monoglycerides. While the absorbents are costly and create a waste stream of spent absorbent, they allow considerable savings in water treatment costs and eliminate the challenges associated with emulsion formation during water washing.

5. Additization

Biodiesel properties may be enhanced by additives. In order to provide acceptable cold flow properties, biodiesel is generally produced from feedstocks that contain substantial amounts of unsaturated fatty acids. These compounds are prone to oxidative attack from oxygen in the atmosphere. Some feedstocks, such as soybean oil, contain potent natural antioxidants such as tocopherol that protect the fuel for six months or more. In cases where extended storage is expected, an antioxidant additive such as tert-butylhydroquinone (TBHQ) is used, frequently accompanied by a metal chelator such as citric acid. Pour point depressant additives are also available for biodiesel but these have only been found to be effective in blends of biodiesel with petroleum-based diesel fuel and are generally added after blending by the distributor or the consumer.

6. Conclusions

Biodiesel is an alternative fuel that is widely used in Europe and is attracting rapidly growing interest in the United States and other parts of the

world. In the U.S., production capacity is increasing very rapidly due to federal government tax incentives and high petroleum prices.

Production of biodiesel utilizes very well-known reaction and separation processes. Some variations exist between plants, but most of the processes are similar. While future developments, such as heterogeneous catalysts, will certainly make an impact, processing is likely to follow well-accepted procedures with increasing commonality between plants in a manner that is similar to that currently found in the fuel ethanol industry.

References

ASTM, Standard Specification for Biodiesel Fuel (B100) Blend Stock for Distillate Fuels. ASTM D 6751-02, American Society for Testing and Materials

Canakci M, Van Gerpen J (2001) Biodiesel Production from Oils and Fats with High Free Fatty Acids, Transactions of the American Society of Agricultural Engineers vol 44 no. 6: pp 1429-1436

Erickson DR (1995) Bleaching/Adsorption Treatment. In: Erickson DR (ed) Practical Handbook of Soybean Processing and Utilization, AOCS Press, Champaign, IL

Freedman B, Pryde EH (1982) Fatty Esters from Vegetable Oils for Use as a Diesel Fuel, from Vegetable Oil Fuels. In: Proceedings of the International Conference on Plant and Vegetable Oils as Fuels, ASAE, St. Joseph, MI

Knothe G, Steidley KR (2005) Lubricity of Components of Biodiesel and Petrodiesel. The Origin of Biodiesel Lubricity. Energy and Fuels 19: pp 1192-1200

Peterson CL, Feldman M, Korus R, Auld DL (1991) Batch Type Transesterification Process for Winter Rape Oil. ASAE Transactions, vol 7 n.6: pp 711-716

Van Gerpen JH, Dvorak B (2002) The Effect of Phosphorus Level on the Total Glycerol and Reaction Yield of Biodiesel. Bioenergy 2002. The 10th Biennial Bioenergy Conference, Boise, ID

Zehnder CT (1995) Deodorization. In: Erickson DR (ed) Practical Handbook of Soybean Processing and Utilization, AOCS Press, Champaign, IL

11

Bioelectricity and cogeneration

Giuliano Grassi, Alexander Allan

EUBIA - European Biomass Industry Association, Rue d'Arlon 63-65, B-1040 Brussels, Belgium

1. Generalities

In general the interest for biomass production and utilisation (and in particular for co-generation) is growing considerably because of the diffuse awareness of the socio-economic, political and environmental benefits.

Actually intensive land use in the E.U. for food crops is decreasing due to the evolution of the Common Agriculture Policy, liberalisation of the Agriculture Markets (i.e. W.T.O), enlargement of the E.U. towards East European Countries most of these still with a significant agriculture economic basis.

In this context it is estimated that more and more good Agricultural Land (S.A.U.) and marginal lands will be available for the production of Energy Crops for potential large scale supply of clean biomass resources.

The present insecurity on future and large-scale conventional energy-supply, pressure for environmental protection, increasing unemployment in rural areas, makes more attractive the exploitation of the huge biomass world-wide potential as new energy vector.

Furthermore the technological development so far reached is able to overcome most of disadvantages related to the biomass utilisation, like:

- Dispersed resource characteristics;
- Seasonal production;

P. Ranalli (ed.), Improvement of Crop Plants for Industrial End Uses, 291–305.

- Low bulk density;
- Low energy density (due to its high moisture content);
- Fast bio-degradation of biomass (with material loss) if moisture level is ≥10%;
- Logistics problems for large volume supply;
- Different chemical composition especially (ashes, micro-elements, heavy-metals.

This chapter reviews the state of art and the development in progress for the electricity production from biomass and in particular interest for cogeneration.

In the following pages an estimation of the biomass potential, on the vital future role of dedicated energy-crops, their performances and characteristics will be presented as answer to the two main questions that are frequently posed:

- How much is the technical EU and world potential of biomass resources and its expected evolution?
- How much biomass resources will be available in particular for the power/cogeneration market in the E.U. and at world level?

In the figure here below, an answer to the first questions is presented:

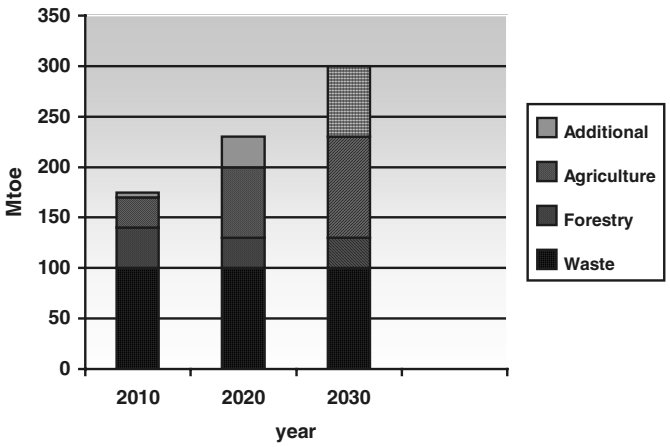


Fig. 11.1. Primary biomass potential (E.U) (E.E.A.ISSN 1830-2246/02-2005) Source: G. Grassi, EUBIA.

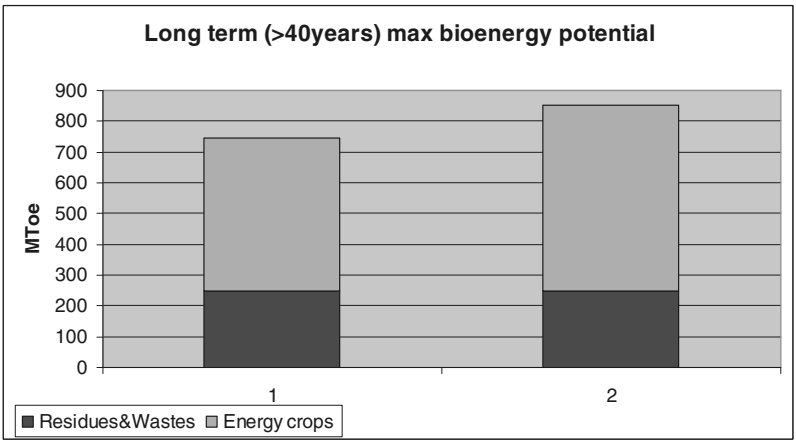


Fig. 11.2. Long term (>40 years) max bioenergy potential. Source: University of Lund, Sweden.

Table 11.1. Projections of future biomass Contribution (M Toe / year).*

Organization	**2025** (year)	**2050** (year)
Shell (1996)	2,030	4,750
IPCC(1996	1,720	6,700
Greenpeace (1993)	2,720	4,320
Johansson et al. (1993)	3,470	4,920
WEC (1993)	1,400	3,000
Dessus et al. (1992)	3,220	-
Lashof and Tirpak (1991)	3,100	5,130
Fisher and Schrttenholzer (2001)	8,350	10,750
Average	3,250	5,650

*Source: White Paper G8 Initiatives on Bioenergy Partnership (2005)

Biomass can be utilized directly for the production of heat, for power generation and for transport bio-fuels. Due to the high increase of electricity demand there is now a particular interest for promoting the production of green power from renewable sources and for bio-electricity.

The estimated future market volume of bioelectricity in the E.U. and world-wide are summarized in the following figures:

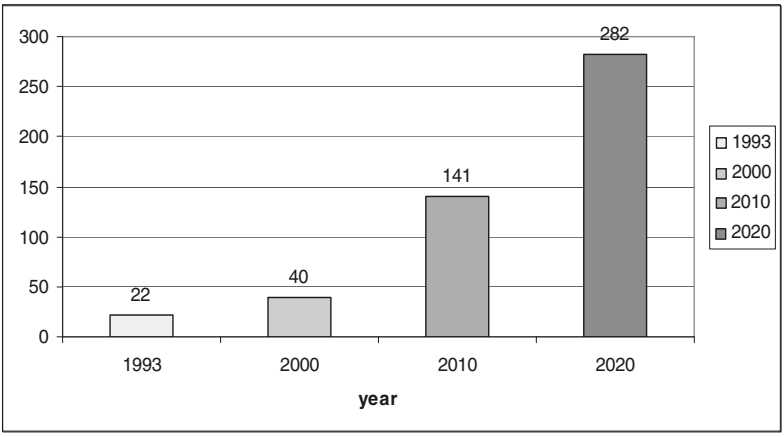


Fig. 11.3. Evolution of future Bioelectricity production**.** Source: G. Grassi EUBIA.

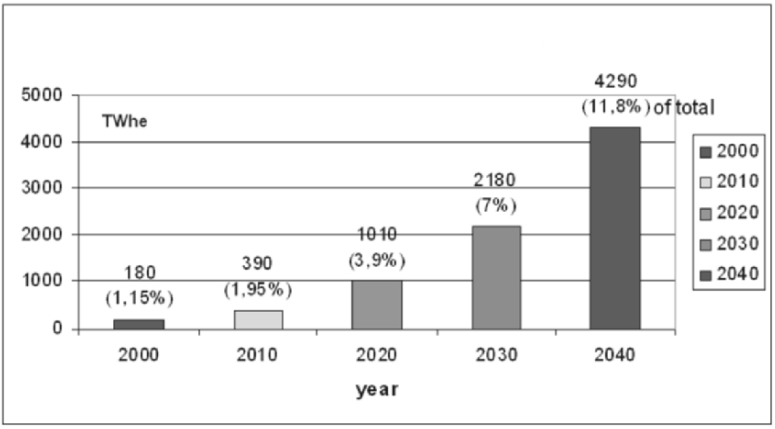


Fig. 11.4. World Bio-electricity Market. Source: G. Grassi EUBIA.

2. Role of energy crops for Bioenergy Markets and Cogeneration

For bioelectricity production, all type of solid, gaseous, liquid biofuels can be utilised, but lignocellulosic biomasses should is seen as the most important biomass resource, because widely available and readily utilisable for combustion, without the need of adopting sophisticated expensive conversion processes.

Three are the main sources of solid biomass:

- Agriculture residues (1.5 billion fresh t/y in the E.U.-25);
- Forestry residues (~250 million dry t/y in the E.U.-25);
- Energy crops.

Extensive testing had been carried out in the E.U., USA etc. during the past 20/30 years on several promising high-yield energy crops in particular high yield C-4 crops.

In the table here below, main experimental data concerning yield and production costs in E.U. are presented:

Table 11.2. Typical Production cost of Energy Crops (trials 2002)* Source: G Grassi EUBIA.

Energy crops		**Yield dry t/ha**	**Production cost€/ha**	**Average biomass cost €/dt**
Sorghum	C4**	25	850	32
Cynara Cardum	C3	10	500	50
Miscanthus	C4	22	650	30
Arundo Donax	C3	30	1100	37
Poplar (SRF)	C3	15	720	48

*****All data have been prudentially decreased of 20% to take the expected differences between experimental and large field productivities.

******C4 crops are herbaceous pants have reduced photorespiration losses (high dependent oxidation process) and higher yield of C3 plants.

For general economic evaluations, the following average productivity and cost values can be assumed for the EU:

- yield of C4 crops: 25 dry t/ha x year (~10 TOE/ha x year);
- yield of C3 crops: 15 dry t/ha x year (~6 TOE/ha x year);
- production cost for C4 crops: 35 (max 50 €/dt);
- production cost for C3 crops: 50 (max 70 €/dt).

From the farmer interest point of view the present Net Income (Production sale value - Production cost) of lignocellulosic biomass (Arundo donax, Miscanthus, SRF Poplar) is around 650 €/ha. The agronomic cultural costs of energy crops are similar to those for growing cereals or animal feeds.

For combustion or power generation important characteristics of energy crops to be taken into account are: their heating values, their chemical composition and the amount and melting temperature of ashes. Typical values are presented in the following tables:

Table 11.3. Technical characteristics of energy crops.

Type of biomass	Chemical content (% wt)					Ashes (%)	Volatile fract. (%)	Melt. Temp. (°C)	L.H.V Kcal /Kg
	C	H	O	N	S				
Sorghum bicolor	40,3	7,6	51,4	0,5	0,1	5,6	74,5	1030	17.181
Miscanthus Sinensis	42,5	7,6	49,7	0,1	0,1	2,8	77,2	1004	17.684
Arundo-donax	42,7	7,5	48,7	0,8	0,2	5	75,2	1016	16.800
Cynara Cardunculus	39	6,6	52,8	0,5	0,2	13,9	72,9	1221	16.781
Poplar SRF	50.90	6.6	42.0	0.2	0.02	0.30	84.0		18.273
Mineral coal	74,2	4	19,8	1,3	0,5	14,2	24,4	1180	27000

Source: Bioenergy Farm - Arsia 6/2004-ISBN 88 82 95 063 8

In summary, Energy Crops will be able to provide a significant contribution to the EU-25 energy needs, considering the large areas of Agricultural lands no more required for food production: 30-40 million ha.

Assuming an average yield of 8 TOE/ha (herbaceous/ Woody Crops) the biomass potential supply for energy from dedicated crops could reach in the E.U. the considerable level of:

~250-320 MTOE/year

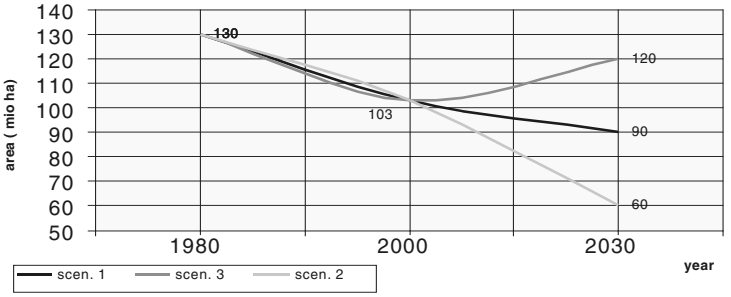


Fig. 11.5. 2000- Projections of agricultural land use in Europe (EU-15) Source: G. Grassi EUBIA.

3. Complexity of the Bioelectricity sector

A very wide range of possibilities for power production and cogeneration from biomass resources is conceivable. Of course, technical-economical-environmental constraints limit actually these possibilities to few practical technologies and systems.

In table here below a general matrix of possible biomass power-cogeneration concepts are summarised:

Table 11.4. General matrix of possible biomass power cogeneration concepts.

Matrix of biomass power generation system						
Type of biofuels	**Steam condensing generators**	**Engines generators**	**Gas Turbine generators**	**Stirling generators**	**Steam generators**	**Gasifer engine generators**
Solid biomass						
biomass	x	-	-	x	x	-
chips	x	-	-	x	x	x
agro-pellets	x	-	-	x	x	x
charcoal	x	-	-	x	x	x
torrified biom	x	-	-	x	x	x
Liquid biofuels						
bioethanol	x	x	x	x	x	-
biomethanol	-	x	x	x	x	-
vegetal oil	-	x	x	x	x	-
bio-crude oil	x	-	-	x	x	-
bio-slurries	x	-	-	x	x	-
Gaseous biofuels						
LHV gas	x	x	x	x	x	-
MHV gas	x	x	x	x	x	-
Bio-hydrogen	-	x	x	x	x	-
Bio-gas	x	x	x	x	x	-

Source: G. Grassi EUBIA

From this wide range of possible scheme, for power-cogeneration production from biofuels, we have summarised in Table 11.5 a limited but practical list of present available and commercial plants.

Table 11.5. List of commercial plants in the U.E.

Commercial Biomass Power Generators (Co-generators)				
Technology	**Capacity**		**Specific investment**	
	Small (kWe)	Large (MWe)	Small (€/kWe)	Large(€/kWe)
Steam condensing (Humid biomass/pellets)	200 - 2,000	Multi	2,000	1,600
Gasifer+Engine	70 - 1,000	-	2,300	-
Biogas+Engine	100 - 600	-	1,800 - 3,000	-
Bioethanol+Engine	20	-	100,000	-
Biethanol turbine	100	50 - 200	1,000	600
Steam engine cogen-erat.	50 - 1000	-	1,300	-
Biohydrogen fuell-cell	10 - 1,000	-	3,000	-

Source: G. Grassi EUBIA

The above table put in evidence the present technological limitation for the bioelectricity production, which are:

- Lack of small capacity, efficient and commercial solid biomass power or cogeneration systems. This type of plant is expected to become the most attractive (especially for cogeneration) because solid biomass is available everywhere it is the cheapest biofuel.
- The TAR gas-cleaning is still not yet a solved problem in biomass gasification, making the use of present sofisticated engine technology no reliable for power generation.
- The life time operation of most present small engines, fuelled with liquid biofuels (bioethanol, vegetal oil, etc.) is limited to about 3,000-30,000 hours against the desirable level of about 100,000 hours.
- Commercial gas-turbine system, fuelled most with natural gas or fuel-oil, need some adaptation for use with liquid or gaseous biofuels.

As far as concern the present "economic limitations" for biomass power production these are due to:

- High specific investments of present commercial technologies due to the limited market volume;
- High cost of biofuels production cost, for example:
  - Agro-pellets.....~ 100 €/t = 250 €/TOE
  - Bio-ethanol.....~ 500 €/t = 700 €/TOE
  - Bio-diesel.....~ - €/t = 800 €/TOE
  - Bio-hydrogen.....~ 2,000 €/t = 670 €/TOE

- Limited electrical efficiency of present technologies
  Most of the electrical efficiency in the bioelectricity sector is actually in the range of 20% to 33%, except for the more sophisticated and expensive IGCC plants.

Of course, cogeneration becomes of great economic interest because, providing a significant supplementary income from the scale of the co-produced heat (in general 2-2,5 times the volume of the power production) can reduce considerably the cost of bioelectricity, especially if the utilisation period of heat can be extended beyond the usual 6 months/year period. This could be the case of industrial utilisation of steam heating and cooling in buildings etc.

4. Stabilisation of humid biomass for power generation

All biomasses at harvesting or at recovery (residues) present an high moisture content, between 25% to 50%. Humid biomass not only has a lower heating value, but also is submitted to a fast biodegradation process with important material loss and noxious emissions into the environment.

In general, for stabilisation of humid biomass "drying by heating" up to 10% moisture level is usually adopted, but this operation requires high investments and operating costs with significant energy consumption equivalent to 20% - 25% of the resource energy value.

However the reduction of the moisture content of biomasses to 10% is important in particular for power generation (steam condensing plants) because the much higher boilers efficiency and heating value of biomass.

Now this advantage can be materialised by the utilisation of an innovative technology able to carry out: "*Simultaneous mechanical drying & compactation (pelletisation) of humid biomass or mixtures*" with low energy input corresponding to about 13% the resource to be refined.

In fact an energy input of 220 kWhe per ton of pellets produced is required for the extraction of ~40% of moisture and its compactation into pellets (6-8 mm) with a moisture content of ~10%. Because in present commercial plants the moisture reduction is ~15% - 20% for one process-step, when biomass has an high humidity content (i.e. 50% -60%) biomass must be circulated back two-three times across the refining dials with loss of time and loss of processing capacity. However multi-stage units are under consideration to overcome this penalisation, of course with additional costs.

In practical terms, Biomass with a moisture level of 50% requires for its conversion into pellets (ø=6mm – 10% moisture) a total energy consumption of ~13% of the output (130kg of pellets for production output of 1000 kg/hr).

Just to appreciate the interest and this new resources saving technology in the refining of humid biomass we summarise in figure here below the processing total resource loss of the new and of conventional technologies:

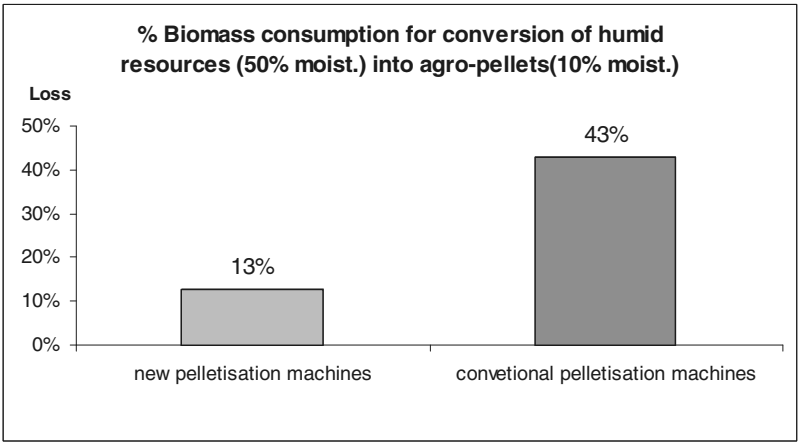


Fig. 11.6. % Biomass consumption for conversion of humid resources (50% moist.) into agro-pellets (10% moist.). Source: G. Grassi EUBIA.

The biomass pellets produced by this new technology have maintained stable their original characteristics for one year (tested interval).

More than 60 different types of humid biomass or mixtures have been tested so far (enclosing high density wood, peat, etc.).

5. Economic aspects

Another peculiar characteristics of this new technology is also related to the possibility of producing pellets from any ***mixture*** of biomasses, without the need of binding compounds.

This aspect is of great interest for reducing the level of ashes or of some noxious micro elements (i.e. Cl, Nx,….) in the final product.

For example agro-pellets obtained from a mixture of 50% of fiber sorghum and 50% of poplar (SFR) shows a considerable reduced (ash)

content from 5,46% to 3,59%, its chlorine content from 180 mg/Kg to 29 mg/Kg, its Potassium content from 792 mg/Kg to 286mg/Kg and its siliceous content from 1,070 mg/Kg to 467 mg/Kg.

Here below, some examples of agro pellets characteristics obtained (Centro Avanzi of Pisa, Italy) from energy crops, from mixtures of biomasses (Poplar-Sorghum) and from typical South Europe agriculture residues:

Table 11.6. Technical characteristics of pellets obtained from different biomaterials.

Type of biomass	**LHV**	**Volatile fract.**	**Dry residue**	**Composition**			
	kj/kg	**g/kg**	**g/kg**	**Silicon**	**Potassium**	**Calcium**	**Chloride**
Cynara Cardunculus	16781	144	71,4	157	1349	1100	1157
Arundo-donax	16800	93,9	37,7	547	385	101	53
Miscanthus S.	17684	219	13,8	7673	7	24	7
Sorghum	17181	105,5	74,6	2380	1059	513	343
Poplar SRF	18273	128,5	25,6	246	95	197	26
Poplar + sorghum	**17250**	**117,2**	**117,2**	**467**	**286**	**194**	**29**
Peach thinning	17892	91,6	91,6	23	127	250	-
Olive thinning	18054	95,4	95,4	77	262	407	3
Vygnard thinning	17960	97,5	97,5	77	195	221	-
Alfa-alfa grass	17295	144,4	144,4	224	445	464	161

Source: G.Grassi EUBIA

Taking into account that the competitiveness of power generation is in general difficult for the motivations listed in the previous chapter, however there are some specific case where the production cost are reasonable and acceptable also in the absence of specific support measures like the "Green-Certificates".

1st Example: *Coal- Agropellets Cofiring*

The utilisation of biomass with mineral coal in thermal power plants has great relevance for the E.U. and in the world.

In particular the estimated potential in the (EU-25) is:

- Bioelectricity capacity is ~30,000 MWe;
- Green-power production volume ~210 TWe/y;
- CO_2 saving volume ~ 150 million t/year.

The specific investment for the adaptation of a coal power plant to a biomass-cofiring operation is very modest: ~40€/KWe installed with a supplementary specific investment of ~200 €/KWe for the integration of the new agro-pellets technology; however this cost should be rather associated to the biomass production & supply sector.

The average estimated production cost of green-power power plants is showed in figure 11.7.

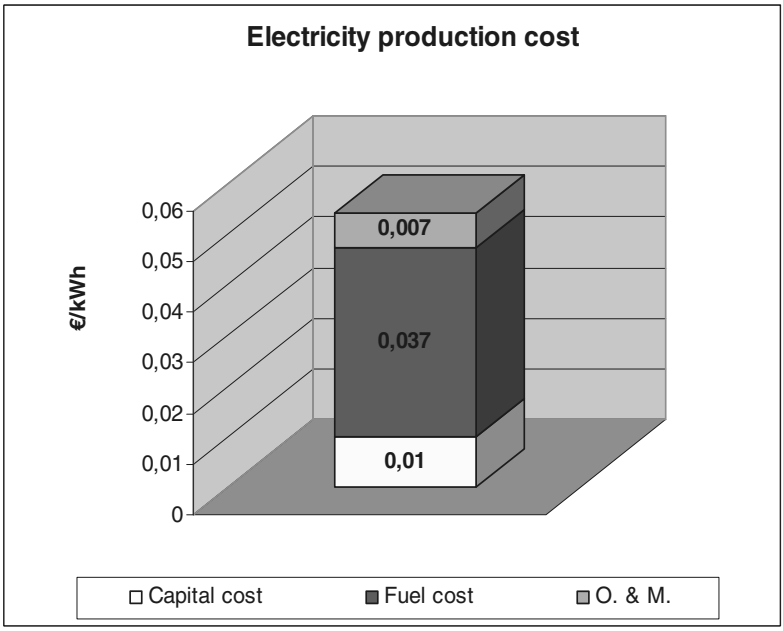


Fig. 11.7. Electricity production cost. Source: G. Grassi EUBIA.

The anticipated total cost is 0.054€/KWhe.

2nd Example: *Small capacity Power-Cogeneration System*

For competition small/medium capacity *cogeneration* units become of considerable economic interest, because the utilisation/sale of the waste-heat (2-3 times the volume of electricity) can be extended beyond the usual 6 months/year period (winter).

This could be the case also of industrial utilisation of steam heating .

Here below some examples of typical economic power-cogeneration evaluations are presented:

Table 11.7. Bioelectrity production costs (€/Kwhe).

Cost of Agro-Pellets	Cogeneration systems (heat sale)	Stand alone Power Generators (not heat sale)
100€/t (no carbon credits)	**0.105** (sale of 5/7 of heat at 0.02€/KWhe)	0.124
40€/t (carbon credit at 40€/t CO2)	**0.048** (sale of 5/7 of heat at 0.02 €/KWth)	0.067

Source: G. Grassi EUBIA

The effect of the availability of CO_2 Carbon-Credits is economically more significant of the benefits driving from the sale of the Cogeneration heat!

To appreciate the CO2 environmental benefits deriving from the substitution of biomass to conventional fuels in power generation, the emissions from different large commercial power generators have been summarized in table below:

In future the general availability of CO2 credits will have large economic benefits for the use of biofuel also for the sectorial power market.

It is useful also to remind that supplementary economic benefits that can derive from the power generation from the integrated full processing of energy crops.

Simultaneous, production of several commodities by combined conversion processes of biomass is a general method for improving the viability of the activity.

Table 11.8. Emissions from different large commercial power generators.

CO_2 EMISSIONS OF ELECTRONIC GENERATION SYSTEM (CO_2 per GWhe)				
	Biomass steam-condensing	**IGCC Plant**	**Conventional steam condensing**	**Nuclear (Boiling water reactor)**
FUEL PREPARATION				5.846
PLANT CONSTRUCTION	0.429	0.429	0.429	0.413
PLANT OPERATION		822.945	1.057.572	6.081
TOTAL	0.429	823.374	1.057.981	12.340

Source: G. Grassi EUBIA

Table 11.9. Emissions from different large commercial power generators.

FUEL	**CO_2 Emissions during combustion (Kg CO_2/Kg fuel)**	**Carbon credit value* for biofuel Substitution (€/t)**
Natural Gas	2.75	
Oil	3.15	
Bioethanol	(2.2)**	88
Biomethanol	(1.7)**	68
Bio-Hydrogen		370
Biomass	0	60
Coal	3.23	

Source: G. Grassi EUBIA

*Carbon credit value ~40€/ t CO2

** But absorbed later by plantations

We can image a:

1. First level of integration consisting for example in a simultaneous production of power & heat (cogeneration).
2. Second level of integration with a simultaneous production of power, heat, biofuels.

The combined production of several commodities, beyond the improvement of the viability, through the exploitation of all components of crops, makes less vulnerable the activity to possible specific markets fluctuations. For example assessments studies on bio-energy complexes based on the production of " Heat + Power + Bioethanol + DDG (grain derived grains)" from sweet-sorghum anticipate a R.O.I (return of investment) in the E.U. conditions around 15% to 25% depending of the size of the plantation, the location and availability of some support measures.

6. Conclusion

Soild Biomass and biofuels represented a good energy resource for energy and in particular for production. Beyond the utilisation of agro-forestry residues/wastes the future large-scale supply of biomass (total estimated potential for the EU-25: 600 MTOE/y will be obtained through optimised plantations of energy – crops. A reasonable range of promising dedicated C3-C4 energy crops have already been identified (SRF: like poplar, willows, Eucalyptus, robinia / Herbaceous crops like sweet: –sorghum, miscanthus, cardum…) with promising development now under way.

Numerous possible schemes for power production (cogeneration) have been investigated in industrialized and emerging countries. However technological problems still limits this wide range to few reliable practical possibilities. CO_2 emission credits or better the "green certificates" make viable in general the production of bioelectricity.

The adoption of cogeneration when is possible at least for the utilisation of wastes heat with several months of operation is able to improve considerably the viability of power production.

12

Genetic improvement of crops for energy generation: comparison of different provision chains with respect to biomass and biofuel production

Paolo Ranalli, Mario Di Candilo

Istituto Sperimentale per le Colture Industriali, Via di Corticella 133, 40128 Bologna, Italy (e-mail:p.ranalli@isci.it)

1. Introduction

In the next 10 to 15 years a number of future biofuels might potentially come on the market. In this paper we will discuss the supply chains of the most promising biofuels, i.e. Ethanol and ETBE from lignocellulosic (woody) biomass; Fischer-Tropsch diesel from lignocellulosic biomass; HTU diesel. Compared with current biofuels, these new products are expected to show superior performance in terms of cost, environmental impact and socio-economic effects.

All the processes for future biofuels that are currently under development still face technical problems and high costs. Only basical insights generated by research and experimental efforts provide opportunity that improvements can be made at every step of the supply chain. Gains in efficiency and cost reductions can be expected when system adjustments will modernize commercial production.

Table 12.1 shows the potential future biofuel routes which are considered in this report. The fossil fuel component, which is likely to be replaced by the various biofuels, has been included. Most of these petrol and diesel substitutes can either be used as a pure, 100% fuel or blended with

P. Ranalli (ed.), Improvement of Crop Plants for Industrial End Uses, 307–334.

Table 12.1. Potential future routes included in this report.

Conversion routes not yet commercially available	Fossil fuel counterpart
Bio-ethanol	
From cereal residues	Petrol
From residues containing lignocellulose	Petrol
From cultivated, short-cycle wood	Petrol
Ethyl Tertiary Butyl Ether (ETBE)	
From bio-ethanol	fossil MTBE / petrol
Hydrothermal Upgrading (HTU)	
From residual organic feed with high water content	Diesel
Biomass FT-diesel[1]	
From dry residues	Diesel
From cultivated, short-cycle wood	Diesel

[1] FT stands for the Fischer-Tropsch process, in which syngas is converted into diesel fuel and naphtha (basic petrol), among other products. Syngas is the product of a biomass gasification process

fossil fuels. In some cases engines need to be adapted if the biofuel percentage is higher than 5-15%, or fuels need to be modified to meet the current fuel specifications.

2. Biomass supply

Many common issues can be discussed at a general, i.e. non specific level as all biofuels require biomass and several biofuels use similar or even the same type of biomass. The different types of biomass, used for the production of different biofuels are:

- Residues (from the food, beverage or fodder industry, or organic wastes from households);
- Food crops (like rapeseed, sunflowers, cereals or sugar beet);
- Short-cycle wood and other lignocellulosic biomass (such as poplar, willow, Robinia, eucalyptus, Miscanthus, giant reed, etc.).

The biofuel provision chain faces different processes which need to be made more efficient, i.e. crop production, harvesting and logistics, pretreatment, handling and standardization and biomass processing from crop into fuel.

A major motivation for quality assurance is to fulfill legal limit requirements (e.g. emission limits), or to meet technical demands for the power plant (e.g. avoiding corrosion). To guarantee these basic conditions, chemical and/or physical parameters may be varied theoretically by crop production processes (e.g. modifying the total nitrogen of whole grain crops by Nitrogen fertilization) as well as by harvesting/preparation (e.g. changing wood moisture content through storage).

3. Existing crops

Existing food crops have a number of disadvantages as energy crops. Most of them are annual crops which require large inputs of energy in cultivation and planting each year (Hulsbergen et al. 2001). A great deal of the yield increase, achieved over the past 50 years, results from the improved partition of total biomass into grain to respond to added nitrogen. Since the entire aboveground portion of the plant is used for combustion, partition is of little relevance for an energy crop. A key consideration for the fuel crop system is their "energy balance"(energy in/energy out). The sustainable use of plants as an energy resource requires a substantial net energy gain.

Simulations that take into account all inputs in the plant-based energy generation process tend to show that the net gain currently ranges between negative and a factor two compared with input energy. This is by far insufficient to play a role of importance in resolving future energy demand. The challenge is to focus on the most sensible ways of producing certain types of energy, and to radically reduce the energy input requirements for growing and harvesting biomass, while maximizing energy retention. The ultimate application of this know-how would be the development of an economically competitive, net energy producing system for the energy sector.

In the plants for the future designed by the Strategic Research Agenda promoted by EU, the milestones of the proposal take into account the following points (EU 2005):

- Considering that the worldwide total energy consumption in 2003 was 6.3×10^{17} KJ.
- Assuming that (i) the global energy demand does not increase, (ii) 10% of the current energy demand will be produced by plants growing on 25% of the arable land (in total 1.4×10^{9} ha), (iii) the energy input for crop cultivation is zero, and (iv) the conversion efficiency from the bio-fuel energy to transportation energy, electricity, or heating is 100%, the annual net energy production should be in the order of 1.8×10^{8} KJ/Ha.

- This yield would require an average oil production of 4.5 ton/ha or an average ethanol production of 6.7 ton/ha per year. These production levels can now only be achieved through high yielding oil or sugar crops, such as oil palm (8 ton of oil/ha) or sugar beet (6 ton of ethanol/ha).

In reality, global energy demand is likely to increase. Furthermore, the contribution of plants to the total energy demand may need to exceed significantly the 10% level, while the land available for energy production is likely to be significantly less than 25%. Moreover, a large part of the available land is not suitable for the current high-energy production systems and cultivation, harvesting and conversion of biomass into energy. This combination of factors will lower the net production of energy. These considerations show that the contribution of plants to the world energy production requires a dramatic increase in production capacity. This requires solutions that go beyond traditional crop production methods. Energy production target levels may be as high as 2×10^9 KJ per ha (equivalent to 50 ton of oil or 75 ton of ethanol per ha per year). This target looks very ambitious but is not unrealistic. The challenge is to develop plants producing a high amount of bio-energy under different climatic conditions.

4. Which qualities are required for an "ideal" fuel crop?

Briefly, an ideal fuel crop should have a sustained capacity to capture and convert available solar energy into harvestable biomass with maximal efficiency and minimal inputs and environmental impacts. In particular this crop has to provide:

- *Maximum light-use efficiency.* The biomass yield limit is set by the available amount of light, its efficiency of interception and the efficiency with which intercepted light is converted into biomass.
- *Water use efficiency.* Available soil water is a significant limitation to crop production over much of N. America and Europe, and irrigation requires significant inputs of energy whilst placing a demand on diminishing water resources.
- *Nitrogen and nutrient use efficiency*. Nitrogen use efficiency is determined at three levels. First, by maximizing the efficiency of energy transduction into biomass in photosynthesis per unit of nitrogen invested in the photosynthetic apparatus. Secondly, by maximizing the amount of N, and other nutrients, translocated out of the canopy components on their senescence, either into other leaves or storage organs; i.e. efficient internal recycling. Thirdly, by

maximizing the capture of soil nutrients. This property will help to minimize both the quantities of N that need to be applied as fertilizer and the amount lost to drainage water.

- *Cultivation, and disease and pest control*. Cultivation operations including ploughing, planting and chemical applications all represent energy inputs; fuel crops therefore need a form and life cycle that minimizes the need for these operations. Energy efficiency and environmental sustainability will be facilitated by selecting crops resistant/tolerant to biotic and abiotic stress with a minimum need for pesticide, fungicide and herbicide applications.

5. The main barriers for progress

It is of crucial importance to set up an action plan that moves from basic research to the development of crops with novel features and to provide farmers with new commercial varieties and optimized farming practices based on specific monitoring tools.

The main strategies for increasing the biomass production can be grouped into four areas:

1. basic plant science e.g., altering plant metabolic pathways to produce certain carbon molecules with valuable functional properties (engineered metabolic pathways to enhance the yield of specific molecules);
2. development of new varieties for specific end uses;
3. production e.g., lowering unit production costs for consistent-quality raw materials (development, production, and handling of crops);
4. processing e.g., more economically by separating different materials (new separations technologies to better handle heterogeneous plant components).

6. Plant metabolism manipulation and exploitation

To improve plant performance it is necessary to explain how synthesis, accumulation and the function of primary and secondary metabolites are controlled.

Primary compounds (e.g. sugars, cellulose, organic acids) provide plants with much of their nutritional and industrial value. Secondary compounds are unique, often species-specific, providing defense against insects and diseases and making plants such a valuable source of new pharmaceuticals. Only a few pathways that produce primary and secondary compounds have

been described; thousands of others remain to be examined. If we can unravel the genetic program of plants for the regulation of the production, storage and use of carbohydrates, lipids and proteins for the food and processing industries, we will be able to i) manipulate the nutritional value and the industrial applications of plant-derived raw, ii) enhance plant defense against pests and parasites, and iii) use plants as bioreactors to produce important plant-derived compounds.

7. Development of elite cultivars for industrial end uses

Different breeding strategies are suitable for different crops. The base of hybrid vigor should be studied in cross-pollinating species, to decipher and exploit underlying mechanisms.

Quantitative trait loci (QTL) analyses has to be performed on specific crops for which the bottleneck productivity may be different.

Once the underlying genes have been identified, their allelic diversity can be exploited. Then, new superior alleles for each of these yield-limiting components could be identified. Advanced breeding strategies would help to combine superior alleles within new crop varieties.

Two parallel strategies should be followed. One uses biotechnological and transgenesis approaches. The other combines the new "Omics" techniques, with traditional breeding approaches, including QTL analysis and marker-assisted breeding. There are potential cross links between these two approaches. Every candidate gene can search for superior alleles within the existing plant material and use a genetic engineering strategy to transfer performing alleles from distantly related species.

7.1 Combining improved photosynthesis and carbon dioxide fixation

Photosynthesis is the primary source of energy for the plant factory and plants have developed specific mechanisms to improve net CO_2 fixation, such as C4 and CAM (Crassulacean Acid Metabolism). The basic cellular processes, involved in the transport of metabolites from source to sink tissues, also needs to be studied. A detailed molecular understanding of these systems and the detection of relatively simple structured variants could result in promising strategies for incorporating such mechanisms in other crop plants with less efficient C fixation or less efficient metabolite production and translocation. This optimization can be achieved by replacing the endogenous sub-optimal alleles with optimized ones. The gene

replacement technologies that enable the targeted modification of existing sequences *in planta* currently not available should be developed.

Plant architecture and developmental characteristics play a critical role in crop performances. The efficiency of metabolite translocation into the "sink" tissues to be harvested is a complex and poorly understood factor which deserves specific analysis, being linked to architectural features. An analysis of sink properties should be performed on the main types of sinks (fruits, monocot and dicot seeds, wood forming tissue, tuber and storage roots) to identify mechanisms controlling these processes and ways to optimize them. This will require a crop-by-crop approach, using, when possible, comparative genomics to speed up the discovery of the genes involved and specific performing alleles.

7.2 Development of new high-energy plant biomass production systems with minimum energy input requirements and higher energy preservation

To achieve plant-based energy production systems which produce the maximum net energy, there is a need to minimize energy input. Current high-energy production systems often require a lot of high-energy demanding inputs, such as fertilizers and pesticides. Strategies to be followed may range from improved nutrient uptake, nutrient use efficiency, or pest resistance for existing high-energy crops, to the implementation of new crops that require fewer high-energy inputs.

The second way of improving the net energy balance of plant-based energy production systems is to increase the energy production levels or the more efficient use of produced biomass A classic example of how efficiency can be improved is the utility of plant biomass for biofuels in the biorefining process. Through increasing the accessibility of the cell wall "energy" polymers of cellulose and lignin to hydrolysis, the sugar production cost should decrease and cheaper sugars should become available for bio-ethanol production.

In existing plant production systems, the level of useful energy produced could be improved by a more efficient conversion of the energy input (through faster plant growth and improvement of photosynthetic efficiency) or an increased storage of the high-energy containing compounds (through optimization of plant architecture or sink-source interactions).

In addition, completely new plant-based energy production systems with maximum energy output need to be developed.

7.3 Improvement of factors contributing to yield stability

Climatic fluctuation can impair a variety of processes, such as rooting, fertilization and grain filling, with important consequences for yield. Plants which are able to withstand drought, cold and salt stress would not only stabilize yield potentials but also contribute to reducing the impact of agriculture on the environment. For example, drought-tolerant crops will require less water for their production and this will lead to reduced erosion and soil salination. These adverse environmental conditions also increase susceptibility to pests and pathogens resulting in a higher consumption of agrochemicals and further yield quality losses. This means that there are direct and indirect benefits to improving tolerance to abiotic factors.

Breeding strategies can reduce the impact of a changing environment on yields.

7.3.1 Improve tolerance to water stress and drought

In many countries, including the European Mediterranean areas, water supply is the most limiting factor. Not only the lack of water but also short periods without rain may affect yield and quality. This means that we need to improve the water consumption efficiency and tolerance to water shortages. Different physiological processes contribute to water homeostasis: root morphology and depth, plant architecture, variation in leaf cuticle thickness, stomatal regulation, osmotic adjustment, antioxidant capacity, hormonal regulation, desiccation tolerance (membrane and protein stability), maintenance of photosynthesis, and the timing of events during reproduction (Bray 1997; Nguyen et al. 1997; Edmeades et al. 2001). Biotechnology is focused on the genetic dissection of drought tolerance through the identification of quantitative trait loci (QTL) associated with yield components, secondary morphological traits of interest, and, more recently, physiological parameters. At the same time the potential of functional genomics is pursued, which should provide useful information about the gene regulation level. Understanding the genetic basis of the essential physiological parameters of drought tolerance, together with the data from profiling experiments, should allow the identification of the key pathways involved in drought stress and how they interact. The emergence of molecular genetics and associated technologies represents a very important new breeding tool; the current challenge is to integrate this tool and the information it generates into breeding schemes to further the development of efficient MAS strategies. These researches are being developed in many laboratories, but their complexity remain a challenge. However, some genes suspected of having an impact on the water consumption efficiency

have already been identified in plants such as sorghum. Some have already been transferred to such crops as maize.

7.3.2 Improved tolerance to low temperatures and frost

Some spring crops do not produce high yields mostly because their vegetative phase is too short. Their adaptation to cold temperatures and frost needs to be improved if they are bred to produce winter varieties.

Significant progress has been made over the past 15 years in understanding the molecular basis of cold acclimation, initiated largely from the study of induced genes (Thomashow 1999). These studies have led to the identification of the CBF transcription factors that are responsible for activating expression of many of the genes induced during cold acclimation in Arabidopsis (Gilmour et al. 2000). Characterization of the closely related (or in some cases identical) DREB transcription factors led to a similar understanding of gene regulation in response to drought stress and also to an appreciation of the mechanistic links between higher plant responses to cold and drought (Seki et al. 2003). The target genes of the CBF transcription factors (cold-induced genes; CORs) provide some clues to the metabolic processes and cellular changes that are important components of acclimation. For example, COR15a is thought to decrease the rate at which the membranes of the chloroplast inner envelope undergo phase transition at low temperatures (Steponkus et al. 1998). Its activity emphasizes that impairment of membranes is one of the most damaging effects of exposure to freezing, and many cellular processes induced during cold acclimation are associated with membrane stabilization.

Plant breeders need to identify single genes that contribute to freezing conditions tolerance and the ability of plants to acclimate to cold. The transcriptional regulators of COR gene expression have provided some potential candidate genes and specific genes have been identified in monocots and dicots (Martin 2004). The way is open for a further increase of the tolerance of different crops by exploiting biodiversity at the corresponding homeologous loci. At the same time, a better understanding of tolerance to freezing at the cell level will help breeding for this trait. The analysis of tolerance process provides great insight into the development of new winter varieties integrating cold tolerance and high yield.

7.3.3 Improving stress tolerance through homeostasis

Homeostasis (tendency to stability): a self-regulating biological process that maintains the stability and equilibrium of the organism.

When a plant is exposed to pathogens, wounding, drought, cold, physical or chemical shocks, survival mechanisms turn on to reduce damage. The balance between stress and survival signals determines the level of damage suffered by the plant. The reactive oxygen species (ROS), among the key molecules studied in relation to stress, have high reactivity and therefore toxicity (such as superoxide, hydroxyl radicals and hydrogen peroxide). Under stress conditions, the steady state level of ROS usually increases, and it has been hypothesized that ROS might also act as messengers turning on stress-related genes (Ron Mittler et al. 2005). Signal transduction pathways that are activated can lead either to stress acclimation or to cell death depending on the degree of oxidative stress experienced. ROS homeostasis in plant tissues is therefore determined by their relative rates of production and destruction. Most stresses interfere with mitochondrial function, deregulates the physiology of the plant and causes NAD^+ breakdown, ATP overconsumption, and enhanced respiration. These reactions deplete the energy of the plant, cause the production of reactive oxygen species, and consequently induce cell death (De Bloch et al. 2005). The NAD^+ breakdown is caused by the enhanced activity of poly (ADP-ribose) polymerase (PARP), which uses NAD^+ as a substrate to synthesize polymers of ADP-ribose. This poly (ADP ribosilation) is a post-translational modification of nuclear proteins that seems to be initiated by oxidative and other types of DNA damage. Plants with lowered poly(ADP ribosil)ation activity appear tolerant to multiple stresses. The researchers demonstrated that inhibiting PARP activity via chemical inhibitors (nicotinamide) or genetic engineering (RNA constructs of the parp1 and parp2 genes) protects plant from oxidative stress and enhanced tolerance to stress, such as heat and drought (De Block et al. 2005).

It can be concluded that, if the inciting stress is not so extreme, plant responses to stress lead to acclimate and repair damage: the enhanced antioxidant turnover protect against oxidative damage and re-establish homeostasis.

7.3.4 Interactions between plants and biotic agents

Interactions between plant pathogens and their host plant are very specific. Often only one plant species or sometimes even only one cultivar of a plant species becomes infected/attacked. Specific molecules or signals are involved in communication between host plants and their pathogens/pests and eventually determine whether a plant becomes infected/attacked or remains healthy.

Various crop protection methods are being used. They include chemical control, resistance breeding and biological control. For an efficient crop protection pathogens should be detected by the plant before or during early

stages of infection. In order to achieve this, sensitive diagnostic tools are being developed to detect particularly viruses and micro-organisms in plants.

For achieving durable crop protection it is important to unravel the mechanisms and genes underlying the specific interactions between host plants and their pathogens. Detailed insight in how pathogens recognize and attack their hosts and how host plants defend themselves provide tools for the development of new strategies to protect the crop plants.

Only in a few cases fundamental research has developed new strategies already. Gene expression encoding various viral proteins in plants protect them against several viruses. This kind of resistance is becoming intensively exploited in engineered resistance breeding. In a similar way studies on the molecular basis of gene-for-gene systems led to a better understanding of how pathogens overcome or avoid resistance in plants. Resistance genes against viruses, bacteria, fungi and nematodes show previously unsuspected homologies which might be exploited to create hybrid resistance genes that can be used in molecular natural resistance breeding.

A common defense response occurring in many plants against viruses, bacteria, fungi and even nematodes and endophytic insects is the hypersensitivity response (HR). However, due to high selection pressures uniform crop plants become susceptible to new variants of pathogens. It is important to understand the molecular basis of this adaptation in order to breed more durable resistant plants. Therefore pathogen's avirulence genes and their complementary natural plant resistance genes or gene clusters are isolated and studied in detail.

Also signal transduction pathways initiated by activation of resistance genes after interaction with avirulent pathogens are being studied. They include locally and systemically induced defense response mechanisms and programmed cell death. In addition, resistance mechanisms are studied that are not associated with HR, such as pre-houstorial and non-host resistance and avoidance. Molecular genetic analyses of these resistances proved that the genes, involved in these resistances, are different from those involved in HR. Detailed studies will give further insight into the function of these genes and the evidence of their durability.

7.3.5 Resistance to pests

Pathogens and other biotic factors, such as pests, are the main constraints affecting yield losses in crop plants. Some of them also have an important impact on quality due to the production of toxins. An obvious alternative to agrochemical protection is the exploitation of naturally occurring resistance mechanisms.

Genes that are used frequently for pest resistance are those encoding proteinase inhibitors (PIs). PIs are proteins that form complexes with proteinases and inhibit their proteolytic activity which are widespread in nature. The observation that plant derived proteinase inhibitors inactivate proteinases of animal and microbial origin, while rarely inhibiting endogenous enzymes, is compelling evidence for the current view that they are involved in the protection of plants against pests, and possibly pathogens (Charity et al. 1999). There is evidence that the introduction of genes that code for proteinase inhibitors into plants can delay the development of feeding insect pests (McManus et al. 1999). However, PIs have demonstrated a significant degree of specificity towards insect pests. This makes it very difficult to use a few genes on many insects. In addition to this, it has been found that different strains of the same insect species may show a differential susceptibility to a specific PI (Girard et al. 1998).

Transgenic plants producing environmentally benign *Bacillus thuringensis* (Bt) toxins are also being used more for insect control, but their usefulness will be short-lived if pests adapt quickly (Heckel et al. 1999). The main strategy for delaying insect resistance to transgenic Bt plants is to provide refuges of host plants that do not produce Bt toxins. This potentially delays the development of insect resistance to Bt crops by providing susceptible insects for mating with resistant insects (Liu et al.1999).

Scientists are trying to insert few genes into crops which should generate resistance to as many pests as possible but they have to face several problems. For example, any kind of insect may be susceptible to a particular proteinase inhibitor, which is being inserted into crops. But the species may not be susceptible to many other proteinase inhibitors. Secondly, different strains of the same species (i.e. taken from different areas) may not be susceptible to the same PI.

7.3.6 Improvement of genetic resistance to pathogens and pests

Fundamental aspects of approach are the multidisciplinary investigation of the epidemiology of pathogens and pests, the mechanisms of plant biotic interactions, the influence of crop management (for example, crop rotations versus monocultures), the search for germplasm resistant to pests, and ways of decreasing the spread of epidemics. All these data should be integrated into modelling studies to define optimal practices to minimize the use of sustainable pesticides (i.e. through precision farming). More important changes can be expected from the development of genomics and plant biotechnologies and their use in breeding programs for the selection of plants which are resistant to pests and pathogens without chemical protection.

The resistance genes can either be new superior performing alleles of already known or existing genes, or completely new genes not present within the particular crop species. They have to be introgressed into the corresponding crop varieties via sexual crossing, if possible, or gene transfer. Conventional resistance breeding often suffers from limited access to suitable resistance sources. The development of gene technology has drastically increased the availability of genes conferring resistance, which can be derived from non-related plant species as well as non-plant sources.

The genetic and molecular dissection of resistance and defense mechanisms are progressing rapidly in model plants. The comparison of the genomes of crop and model species belonging to the same botanical families revealed a high degree of conservation in the genome structure. This conserved synteny will facilitate the transfer of information gained in model species to related crops and the identification of genes and quantitative trait loci (QTL) that control resistance to divers biotic stresses. Natural and induced genetic diversity will be exploited in breeding programs to generate germplasm of resistant plants. It is reasonable to expect that a major outcome of plant biotechnology and genomics during the coming two decades will be the construction of plants resistant or tolerant to the various pests and pathogens that threaten them.

8. Converting biomass into energy

The traditional way of converting biomass into energy is simply to burn it to produce heat. Heat can be used directly, for heating, cooking and industrial processes, or indirectly, for the production of electricity. The non combustion methods convert raw biomass into a variety of gaseous, liquid, or solid fuels before using it. The carbohydrates in biomass, which are compounds of oxygen, carbon, and hydrogen, can be broken down into a variety of chemicals, some of which are useful fuels.

Heat production and electricity generation are the most important uses for biomass fuel worldwide. Direct combustion devices are widely distributed with thermal capacities ranging from a few kW in household stoves up to heating plants with several tens of MW. The conversion efficiencies vary from 8 to 18% for simple stoves used traditionally in developing countries, up to 90% and above for modern heating units with high-end technology. Electricity production is based mainly on the conventional steam cycle with efficiencies around 30% and a capacity of several hundreds of kW and above.

Whatever production system, the supply of sufficient quantities of plant biomass, with the right composition and at competitive price, is a prerequisite to the future success of bio-energy. More in details, conversion can take place in three ways:

I. Thermochemical. When the plant matter is heated, but not burned, it breaks down into gases, liquids, and solids. These products can then be processed into gas and liquid fuels like methane and alcohol. Thus gasification, pyrolysis and charcoal production are all relevant but only charcoal production is currently widely used. Gasification for electricity production seems to be a quite promising option which might become available on the market in the next few years. An option for the future is Pyrolysis, with the aim of providing a liquid fuel useable in power units.
II. Biochemical. Bacteria, yeasts, and enzymes also break down carbohydrates. Fermentation changes the biomass liquid into alcohol, which is inflammable. A similar process is used to turn corn into ethanol, which is mixed with gasoline to make gasohol. Also, when bacteria break down biomass, methane and carbon dioxide are produced. This methane can be captured, in sewage treatment plants and landfills, for example, and burned for heat and power.
III. Chemical. Biomass oils, like soybean and canola oil, can be chemically converted into liquid fuel similar to diesel fuel, and into gasoline additives. The most important process so far is vegetable oil production from oil seed, and the esterification of this oil fatty acid methyl ester as a substitute for diesel fuel. This technology is used on a large scale across Europe.

Biomass is also used to make gas additives like ETBE and MTBE, which reduce air emissions from cars.

8.1 Bio-ethanol

Nowadays bio-ethanol can be synthesized from a wide variety of biomass, as long as it contains readily fermentable sugars or starch. It can be produced from biomass, grown specifically for bio-ethanol production (such as sugar cane, sugar beet or cereals), or by converting by-products (secondary products) from the sugar and cereal industry. In Brazil, the world's largest bio-ethanol producer, sugar cane is used as feedstock. In Europe, bio-ethanol is normally produced from sugar beet and cereals.

At the same time, a number of companies and research Institutes worldwide are working on the further development of bio-ethanol production processes to enable (ligno-) cellulosic biomass to be used as a feedstock. This would create benefits compared to current technology (IEA 2005):

- Access to a much wider array of potential feedstocks
- Less land use conflicts on food and fodder production
- Greater net GHG (Greenhouse Gas) reduction potential
- More fossil fuel replaced

Below, the options in which ethanol is produced from sugar beet, cereals or wheat by-products are first discussed, followed by an analysis of ethanol from cellulosic biomass.

8.1.1 Ethanol from sugar beet, wheat or residual C-starch

Technology

If sugar beet is used for the production of bio-ethanol, the beets are cultivated and transported to an ethanol plant, where the biomass is broken down by fermentation into sugar beet pulp, the by-product, and a water-ethanol mixture. The latter is converted into pure ethanol via distillation. The beet pulp can be used as animal fodder or fuel.

Ethanol can be produced from wheat grains by milling, hydrolysis, fermentation and distillation. The process is more complex and expensive than with sugar beet. Milling and distilling are the most energy-consuming unit operations. In addition, ethanol can be produced from residual organic biomass, for example from by-products of the wheat and sugar beet industry (Kampman 2005), as long as these contain sufficient amounts of readily convertible sugars (C6 sugars such as glucose).

GHG reduction

The calculations in the General Motors-study (GM et al. 2002) regard sugar beets cultivated in a rotational system. The crop residues are ploughed into the soil. Fertilizer use is approximately 100 kg N/ha/year, while the ethanol yield is estimated about 4.800 liters per ha. Two different reference systems (i.e. replaced crops) are considered: Egyptian clover and rye grass. Ethanol GHG emissions are found to be 41-86% of those associated with petrol production, depending mainly on the use of the by-product.

8.1.2 Ethanol from lignocellulosic biomass

Technology

The ethanol production from lignocellulosic biomass requires an additional pre-treatment of the feedstock, which enables the fermentation of sugars contained in the biomass. The fermentation process itself needs to be adapted, furthermore, new kinds of enzymes being required to convert the C5 sugars into ethanol (in the production of ethanol and alcohol today it is only C6 sugars, the main constituent of the current feedstock, that are converted). The process consists basically of four steps:

- pre-treatment. This stage is necessary to make the material accessible to enzymes mediating enzymatic hydrolysis and to break down hemicellulose into C5 sugars;
- hydrolysis of cellulose;
- fermentation of C5 sugars (xylose) and C6 sugars (glucose);
- distillation.

Today's research is mainly focused on three issues: improvement of the pre-treatment stage, integration of hydrolysis and fermentation in fewer reactors to cut costs, and the improvement of the 5 fermentation process (ECN 2003). The progress and success of these developments will determine the future potential of this route and when these biofuels can first be marketed on significant scale.

Pre-treatment

Pre-treatment is necessary to improve fermentation efficiency. The goals of pre-treatment are:

- to make the material accessible to enzymes for hydrolysis, by reducing its volume and opening up the fibrous material.
- to mobilize the lignin and (hemi)cellulose biopolymers and attain the further break-down of structural components to optimise enzymatic access in the following steps.

Several methods are available for pre-treatment: dilute acid hydrolysis, alkaline pre-treatment, steam explosion, liquid hot water pre-treatment.

Bottlenecks for commercial application

Ethanol production from lignocellulosic biomass has several advantages compared with conventional technology: the use of cellulosic feedstock for fuel production, and therefore a better GHG balance; lower feedstock costs because of higher feedstock yields (in MJ per hectare). The drawback is enzyme technology. Cellulase enzymes are produced commercially, but in low volumes geared to high-value products. The industrial cellulases currently available are not effective enough and too costly for use in large scale production (ECN 2003). Besides, pentose can be converted only in

part because there are no fermentation systems available that can use the entire pentose fraction. Different efforts have been made to tackle this problem: genetic modification of backer's yeast; co-culture of different strains of *Zymomonas mobilis*; transfer of pentose-converting genes into ethanol-resistant strains of *E.coli*. Even if some progress is reported in research journal, there is still a lack of experience at the industrial scale.

GHG reduction potential

According to a GM study, GHG emissions of ethanol from wooden biomass crops are 20-30% lower than for ethanol from sugar beet or cereals.

8.2 ETBE

Ethanol can be converted into ETBE (Ethyl Tertiary Butyl Ether), that can be used to replace MTBE, a petrol component. Whereas the MTBE percentage in petrol usually ranges between 1 and 5% (by volume), more ETBE can be added: up to 15%.

ETBE can be produced using any of the previously discussed ethanol variants, regardless of the feedstock or conversion process used. This means two things:

- Nowadays ETBE can be produced from ethanol, using cereals or sugar beet (or secondary products yielded as by-products of the food and fodder industry) as a feedstock.
- The environmental and cost performance of ETBE and its potential availability will benefit from any improvements in the ethanol production process (in particular, the expected development of technology enabling conversion of (ligno)cellulosic biomass).

ETBE, compared to ethanol, can be blended with petrol without changing the vapor pressure (in case of low-percentage ethanol blends). In EU countries like France, Spain and Italy, ETBE has already been added to petrol as an alternative to MTBE.

8.3 Biodiesel

Two sources of diesel substitute, vegetable oil-based biodiesel and synthetic diesel from a lignocellulosic biomass-based Fischer-Tropsch process, are assumed to replace diesel.

All the stages of the vegetable oil-based biodiesel chain can use proven commercial technologies and several European countries have an established biodiesel industry, e.g. Germany, Austria and France (based on rapeseed and recovered vegetable oils).

Significant improvements are expected to affect rapeseed yield and the reduction of energy needs for oil extraction and esterification.

8.3.1 Biomass Fischer-Tropsch diesel

Technology

Fischer-Tropsch (FT) hydrocarbons can be produced by gasification of biomass, followed by downstream gasification. The biomass FT plant comprises: biomass pre-treatment (chipping, drying), gasification (resulting in syngas), gas cleaning and conditioning, FT-reactor, hydro-craker. As with the ethanol production process, different configurations are possible. Most configurations produce electricity and heat as by-products. Overall process efficiencies vary according to the plant design from 40% up to 60-65% (Ecofys 2003).

The Fischer-Tropsch technology is one of the options available for utilizing cellulosic biomass for fuel production. As discussed earlier, this reduces the amount of land needed for biomass production compared with current biofuels. It also leads to cost reductions and thus to more cost effective GHG reduction.

Bottlenecks for commercial application

Several technical limitations still stand in the way of commercial application (Hamelinck 2004). The most critical step is the cleaning of the syngas (mixture of CO and H2). According to Hamelinck it is not clear whether the strict cleaning requirements for biomass FT synthesis can be achieved (some impurities need to be removed down to levels of less than 10 ppb by volume).

8.3.2 HTU diesel

The Hydro Thermal Upgrading process is based on depolymerisation and deoxigenation of biomass by means of hydrolysis and decomposition. The process converts biomass into a “biocrude” using highly pressurized water (100-200 bar) at 300-360°C. This biocrude is non-miscible with water and has a relatively high energy content. For application, two routes are possible:

- Power generation;
- Diesel fuel production by catalytic hydrodeoxygenation (HDO).

Croezen and Kampman (2005) analyzed their potential.

A comparative advantage of the HTU process is that it can process biomass streams with a otherwise limited applications potential that are therefore low-priced.

Whereas most biomass conversion techniques require dry biomass, the HTU process can process wet biomass.

8.3.3 Greenhouse gas (GHG) reduction potential

The General Motors study (GM et al. 2002) provides a comprehensive review of the various steps in the rapeseed biodiesel chain. The study presumes that rapeseed is grown on set-aside land in a rotational system with a rye grass reference system (i.e. the crop that would otherwise have been grown on the land). The GM-study assumes a fertilizer use between about 100 and 145 kg per ha and a crop yield of about 3 tons per ha. N_2O emissions are calculated according to the IPCC method, with average assumptions. Crop residues are ploughed in, as is usual in the case of rape crop. The by-product glycerine will normally replace glycerine produced conventionally in the chemical industry. Using these assumptions and ranges, the GM-study estimated the biodiesel GHG emissions to be 21-73% of diesel emissions. Most of these emissions occur during cultivation of the rapeseed. These results are based on average emission factors for nitrous oxide. In reality, however, greenhouse gas emissions are highly variable, differing per soil type, climate and fertilizer input. They are consequently of major influence on total GHG emissions.

9. Potential future biofuel processes

In the next 10 to 15 years a number of future biofuels may be put on the market. The most promising biofuels are:

- Ethanol and ETBE from lignocellulosic (woody) biomass.
- Fischer-Tropsch diesel from lignocellulosic biomass.
- HTU diesel.

All these potential future biofuels are still under development, with conversion processes not yet fully operational on any substantial scale. Compared with current biofuels, these new products are expected to show superior performance in terms of cost, environmental impact and socio-economic effects. This superior performance derives from new processes being able to convert alternative types of biomass feedstock.

10. Technical, economical and environmental limitations

The potential future biofuels cited above are still in the research and development phase and are not yet available on the market due to technical limitations. Even when these technical problems have been solved, economical limitations could hinder large-scale application: significant investments are still required for developing these new biofuel technologies. At least until these biofuels are marketed on a large scale or the costs of other fuels increase significantly, they are likely to remain more expensive than conventional fossil fuels. Market access will then be dependent on government incentives. In the long term, however, costs are predicted to fall, so that eventually all of the future biofuels discussed here will be able to compete with their fossil counterparts as well as with current biofuels. The realization of this cost reduction will depend on:

- technological developments (resulting in a specific process design, conversion efficiency, etc.);
- biomass prices (which, in turn, depend on competition with other potential users of the biomass or land, such as food or energy sector);
- conversion process operating costs (e.g. cost of enzymes for producing lignocellulosic ethanol and ETBE);
- fossil fuel prices;
- government incentives and policies that promote the development and market introduction of these fuels.

Despite these uncertainties, cost estimates for future biofuels can be found in literature. In Figure 12.1 (Hamelinck 2004) they are compared with existing biofuels, petrol and diesel costs.

We can see that cost estimates are quite similar for all the future biofuels considered. Estimated production costs for HTU diesel are comparable to current diesel production costs if wet organic residues with a negative market value are applied as a feedstock.

11. Potential for net greenhouse gas reduction

Figure 12.2 (Hamelinck 2004) compares the Greenhouse gas emissions of the various biofuels with their fossil counterparts analyzed in this paper.

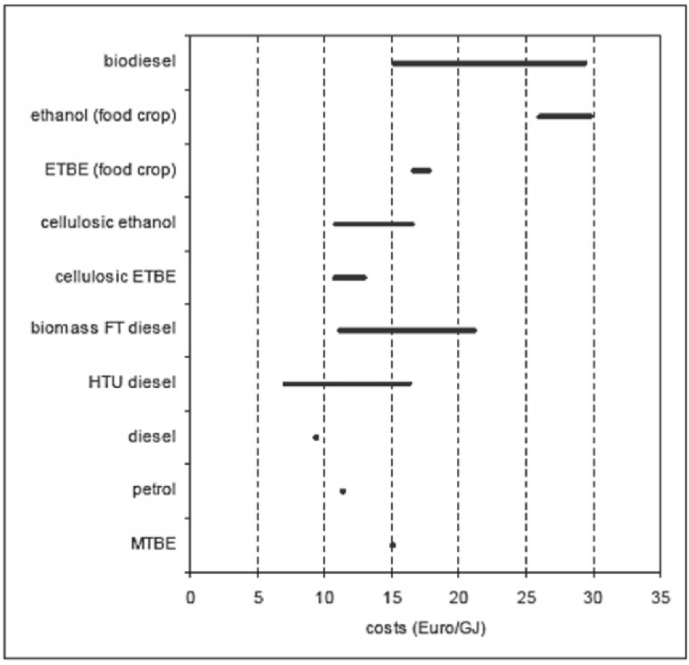


Fig. 12.1. Cost estimates of the various biofuels, compared with the average cost of diesel and petrol in 2002-2004.

Cellulosic ethanol, biomass FT diesel and HTU diesel are expected to produce much higher GHG reductions than currently available biodiesel and ethanol from wheat or sugar beet. Total GHG reductions of over 90% are in fact expected from biomass FT and HTU diesel. Converting ethanol into ETBE has clear advantages from the perspectives of biofuels quality control, but reduces GHG reduction potential significantly because it is produced only partly from ethanol and partly from fossil isobutylene.

The GHG reductions of the various biofuels were found to depend strongly on the emissions associated with biomass feedstock. The superior performance of the observed future biofuels is mainly due to their potential for using lignocellulosic biomass as feedstock.

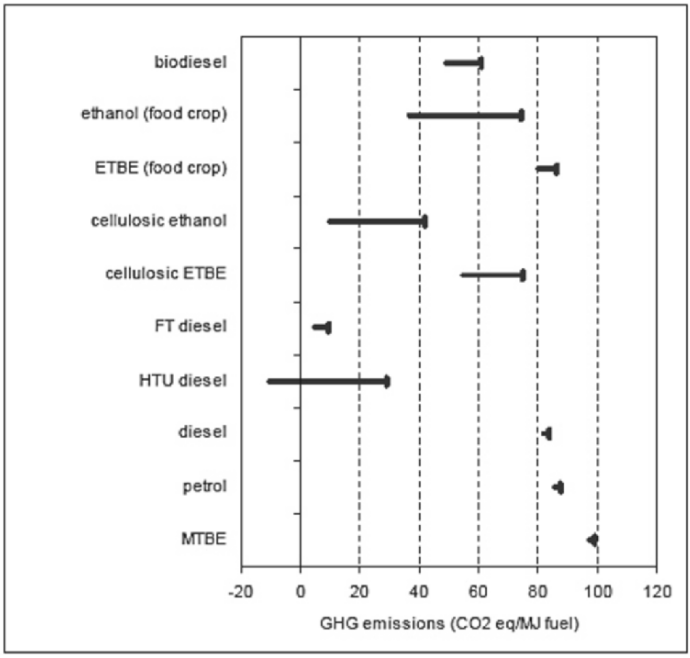


Fig. 12.2. Overview of the GHG emissions of each of the biofuels analyzed, compared with those of diesel, petrol and MTBE (CO2 eq/MJ fuel).

12. A case study

The experiments which were carried out in clayey soil in Bologna (Italy) in the three-years period 2002-2004, compared 7 species, 2 of which annual (*Cannabis sativa* and *Sorghum bicolor*), 2 perennial herbaceous (*Miscanthus sinensis* and *Arundo donax*) and 3 woody species ("*Populus* x *canadensis*", *Salix alba* and *Robinia pseudoacacia*) managed with the method of Short Rotation Forestry (SRF) (Di Candilo et al. 2004a,b).

The woody species were planted at a density of 0.9 plants m^{-2}, with a distance of 1.80 m between the rows and 0.60 m along the rows, whereas the herbaceous perennial were planted with a 0.60 x 0.60 m layout. Hemp and sorghum were sown with distances of 20 and 50 cm between the rows for a theoretical density of 100 and 20 plants m^{-2}, respectively.

Harvesting occurred annually for hemp (first ten days of August), sorghum (first ten days of October), Miscanthus and Arundo (early February). Instead, biomass from woody crops was harvested twice. The first time at the end of the second year (on two-year old trees) and the second at the end of the third year (on one year regrowth).

The parameters assessed were: plant density, plant height, stem diameter, fresh biomass and dry matter production. On the latter were detected net calorific value, ashes, silica and low-melting salt contents.

The thermo-electric conversion of biomass was performed at the Dister power generating plant at Faenza (Ravenna, Italy) which has a capacity of 13.5 Mw. The heating cycle involved the production of steam at 47 bar and 430°C, its subsequent expansion in a turbine up to a counter-pressure of 3.3 bar, stabilization of the steam at 165°C and its distribution to the technological services. The power station is normally supplied with natural gas, biogas, wood-cellulose compounds, such as fruit stones, or spent marc. The boiler can burn three fuels at the same time and fuel changeover can take place without switching off.

Results

Plant emergence for annual species (hemp and sorghum) was very satisfying.

The rooting of cuttings and rhizomes for perennial species was satisfying as well. The crops showed vigorous growth, except for August 2003 when the woody species, especially willow, due to particularly high temperatures and heavy drought, showed an early halt in vegetative growth, immediately restored after the first rain in September.

The mean annual production of fresh biomass was very high for sorghum and Arundo (93.7 and 78.4 t ha^{-1}). Other species showed more modest production levels, between 47.3 t ha^{-1} for hemp and 23.0 t ha^{-1} for Robinia (Figure 12.3).

Arundo provided the highest average annual yield of dry matter (35.4 t ha^{-1}). High yields were also obtained with sorghum and miscanthus (29.7 and 23.3 t ha^{-1}). Hemp, poplar and robinia supplied similar yields, between 15.6 and 13.4 t ha^{-1}, significantly lower than miscanthus.

Willow was found at the bottom of the list due to its sensibility to water stress; in fact, a previous study (Lindroth and Bath 1999) shows that water deficiency is often a growth-limiting factor in willow cultivation.

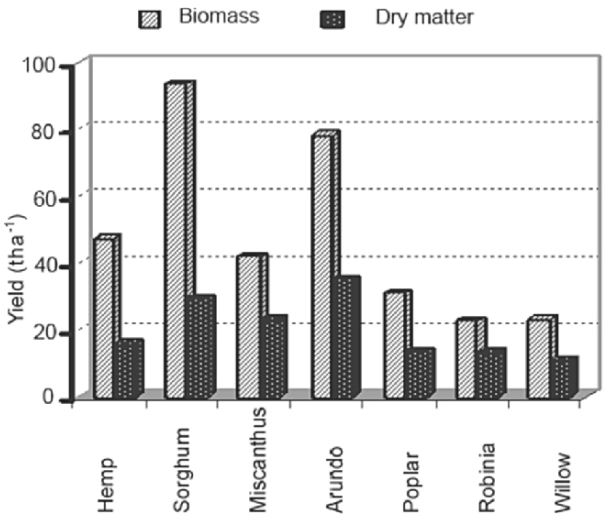


Fig. 12.3. Average annual production of the species compared.

Table 12.2 shows the Net Calorific Value (N.C.V.) and the content of ashes in the dry matter. Robinia, hemp and poplar show the highest values for the first parameter.

Table 12.2. Net Calorific Value and ash contents of biomasses.

Species	N.C.V. (MJ kg^{-1} d.m.)	Ashes (% d.m.)
Hemp	16.4	2.5
Sorghum	14.1	4.5
Miscanthus	14.6	2.6
Arundo	14.6	5.1
Poplar	15.9	2.5
Robinia	16.4	2.5
Willow	15.1	2.6
Means	15.3	3.2

Ash contents in the dry matter were also low in hemp, robinia and poplar. Arundo and sorghum obtained the highest content (5.1 and 4.5%).

Considering biomass production and the net calorific value of the same biomass at the time of combustion in the boiler, the corresponding values in m^3 of natural gas were calculated as well as the equivalent in tons of oil

required per hectare (Table 12.3). The biomass provided by arundo and sorghum was equivalent, after energy conversion, to 14,966 and 12,126 m^3 ha^{-1} of natural gas and to 12.35 and 10.00 t ha^{-1} of oil. The values obtained for SRF were much lower.

Table 12.3. Biomass performance at the combustion in the boiler and energy equivalent in natural gas and crude oil.

Species	Biomass (t ha^{-1})	Moisture (%)	N.C.V. (mj kg^{-1})	CH4 Equiv. (m^3 ha^{-1})	Oil Equiv. (t ha^{-1})
Hemp	18.4	15.1	3.83	7,408	6.11
Sorghum	38.1	22.1	10.85	12,126	10.00
Miscanthus	27.7	16.0	12.16	9,850	8.13
Arundo	44.8	21.0	11.40	14,966	12.35
Poplar	28.2	51.1	7.46	6,354	5.24
Robinia	22.3	40.0	9.59	6,363	5.25
Willow	16.6	35.0	9.66	4,754	3.92
Means	28.0	28.6	10.71	8,832	7.29

Research achievements

- Arundo, sorghum and miscanthus seem to be the most suitable species for the investigated site. Arundo proved to be very rustic, with a strong resistance to pests and lodging, and a good adaptation to dry conditions. These characteristics are fundamental for the good adaptation of species to different pedo-climatic conditions, as well as for a stable yield over time.
- The production levels of SRF were quite modest, compared with the greater pest control requirements (at least for poplar and willow) and water (in the case of willow). For these crops the biennial harvest provided better results than annual harvests.
- The most important factors for the assessment of the species performance are the production of dry matter per unit of surface area and the net calorific value of the dry matter which indicate the total amount of calories that can be obtained from the biomass in the boiler.

13. Coordinated approach and economic sustainability

In order to increase the biomass production we need to develop plants able to tackle biotic and abiotic stress and to provide high performance even in poor areas. The challenge is to modify plat metabolic pathways to enhance the yield of specific molecules.

The future utilization of renewable resources requires a multi-disciplinary, cross-industry approach. Already exciting opportunities exist for research in areas such as processing, logistic, harvesting and new sources of renewable energy. This means that progress in single isolated technical areas will not be sufficient. It will be much more important to have interrelated research projects conducted in a parallel and coordinated manner. The outcome should produce improved fit and flow through the chain, and avoid progress in one area that results in a "surprise" at another point in the system. For example, a scientist may discover a entirely new polymer with functional potential to be the source for an advanced biodegradable plastic replacement. However, the value of this research result is limited until: the appropriate gene is found; it is expressed in the correct viable metabolic pathways; the optimum crop type is grown with enough yield for cost-effective sourcing; a process is created to separate the component polymer; and a method is developed to utilize the material in the manufacturing of the novel product (Zhu et al. 2004).

The sustainability of biofuels also depends on economic aspects. In many cases, the costs for the use of plant-based materials is rather high, and not competitive with fossil fuels. However, cost-competition contains very complex interactions among factors like: product value, material costs, volume of throughput, degree of processing required, and performance of the biofuel generated. Thus, strategies for the future will not be successful if they are based on cost reduction alone.

The most important economic driver is not cost per se, but the difference between the obtained price and manufacturing costs. The obtained price covers aspects such as product utility, performance, and consumer preference and demand. Manufacturing costs cover raw material costs, supply consistency, process required, waste handling costs and investment.

References

Bray, EA (1997) Plant responses to water deficit. Trends in Plant Sci. 2: 48-54

Charity JA, Anderson MA, Bittisnich DJ, Whitecross M, Higgins TJV, (1999) Transgenic tobacco and peas expressing a proteinase inhibitor from *Nicotiana alata* have increased insect resistance. Molecular Breeding 5: 357-365

Croezen HJ, Kampman BE (2005) Duurzame transitie met HTU, een verkenning van de score van het HTU-proces op duurzaamheidsaspecten. CE, Delft

De Block M, Verduyn C, De Brouwer D, Cornelissen M (2005) Poly (ADP-ribose) polymerase in plants affects energy homeostasis, cell death and stress tolerance. The Plant Journal 1: 95-106

Di Candilo M, Ranalli P, Cesaretti C, Pasini P (2004a) Biomass production and energy-transformation trials. In: Proceedings of the 2nd World Conference and Technology Exhibition on Biomass for Energy, Industry and Climate Protection, Rome, pp 1824-1827

Di Candilo M, Ranalli P, Cesaretti C, Pasini P (2004b) Colture non food: ormai realtà l'uso a fini energetici. L'Informatore Agrario 1: 34-38

Ecofys BV (2003) Biofuels in the Dutch market: a fact-finding study. Report 2GAVE03.12. Netherlands Agency for Energy and the environment, European Technology Platform, Utrecht

Edmeades GO, Cooper M, Lafitte R, Zinselmeier C, Ribaut JM, Habben JE, Loffler C & Banzinger M (2001). Abiotic stresses and staple crops. In: Nosberger J, Geiger HH, Struik PC (eds) Crop Science: Progress and Prospects. Proceedings of the Third International Crops Science Congress, CABI, Wallingford, UK

Energy Research Centre of the Netherlands (2003). Ligno Cellulosic-Ethanol. A second opinion. Report 2GAVE-03.11. ECN

EU (2005). Stakeholder Proposal for a Strategic Research Agenda 2025. Part II. pp 102

Gilmour SJ, Sebolt AM, Salazar MP, Everard JD & Thomashov MF (2000) Overexpression of the Arabidopsis CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. Plant Physiol. 124: 1854-1865

Girard C, Bottino M, Deleque MH, Jonuanin L (1998) Two strains of cabbage seed weevil (Coleoptera: Curculionidae) exhibit differential susceptibility to a transgenic oilseed rape expressing oryzacystatin I. Journal of Insect Physiology 44: 569-577

GM, LBST, bp, ExxonMobil, Shell, TotalFinaElf (2002) Annex "Full Background Report" to the GM well-to-wheel analysis of energy use and greenhouse gas emissions of advanced fuel/vehicle systems. A European study, L-B-Systemtechnik GmbH, Ottobrunn, Germany, pp 74-75

Hamelinck CN (2004) Outlook for advanced biofuels. Ph.D. thesis, Utrecht University, pp 232

Heckel DG, Gahan LJ, Liu YB, Tabashnik BE (1999) Genetic mapping of resistance to Bacillus thuringiensis toxins in diamondback moth using biphasic linkage analysis. In: Proc. National Academy of Sciences of the USA 96, 8373-8377

Hulsbergen KJ, Feil B, Biermann S, Rathke GW, Kalk WD, Diepenbrock W (2001) A method of energy balancing in crop production and its application in a long-term fertilizer trial. Agriculture Ecosystems & Environment 86: 303-321

Kampman BE, Croezen HJ, den Boer LC (2005) Duurzaamheid van de bioethanol transitie. CE Rapport, Delft

Lindroth A, Bath A (1999) Assessment of regional willow coppice yield in Sweden on basis of water availability. Forest Ecology and Management 121 (1-2): 57-65

Liu YB, Tabashnik BE, Dennehy TJ, Patin AL, Bartlett AC (1999) Development time and resistance to Bt crops. Nature 400 (6744): 519

Martin K (2004). Cold Tolerance, SFR2, and the Legacy of Gary Warren. The Plant Cell 16: 1955-1957

McManus MT, Burgess EPJ, Philip PB, Watson L, Laing WA, Voisey CR, White DWR (1999) Expression of the soybean (Kunitz) tripsin inhibitor in transgenic tobacco: effects on larval development of Spodoptera litura. Transgenic Research 8: 383-395

Mittler R, Vanderauwera S, Gollery M, Van Breusegem F (2005) Reactive oxygen gene network of plants. Trends in Plant Science 9 (10): 490-498

Nguyen HT, Babu RC, Blum A (1997) Breeding for drought resistance in rice: physiology and molecular genetics considerations. Crop Sci. 37: 1426-1434

Seki M, Kamei A, Yamaguchi-Shinozaki K, Shinozaki K (2003) Molecular responses to drought, salinity and frost: common and different paths for plant protection. Curr. Opin. Biotechnol. 14: 194-199

Steponkus PL, Uemura M, Joseph RA, Gilmour SJ, Thomashow MF (1998) Mode of action of the *COR15a* gene on the freezing tolerance of Arabidopsis thaliana. In: Proc. Natl. Acad. Sci. USA 24: 14570-14575

Thomashov MF (1999) Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. Annu. Rev. Plant Physiol. Plant Mol. Biol. 50: 571-599

Van Tuil R, De Jong E, Scott E, Weusthuis R, Vellema S, De Keizer I, Croezen H (2002) Biomass for the chemical industry. ATO, Wageningen

Zhu J, Shi V, Lee BH, Damsz B, Cheng S, Stirm V, Zhu JK, Hasegawa PM, Bressan Ray A (2004) An Arabidopsis homeodomain transcription factor gene, HOS9, mediates cold tolerance through a CBF-independent pathway. In: Proc. Natl. Acad. Sci. USA, 101, pp 9873-9878

13

Anaerobic digestion: a multi-faceted process for energy, environmental management and rural development

Rudolf Braun

Institut für Umweltbiotechnologie, Interuniversitäres Department für Agrarbiotechnologie - IFA Tulln, Universtät für Bodenkultur, Konrad Lorenzstraße 20, A-3430 Tulln, Austria
(email: Rudolf.Braun@boku.ac.at)

1. Historical development

The biological transformation, by which organic matter is degraded to methane and carbon dioxide is commonly called "methanogenesis". The main product of methanogenesis, a mixture of carbon dioxide and methane, is called "biogas". The term "biogas" was registered as trade name (Institute of Gas Technology, Chicago, United States), but nevertheless, is commonly used by the public.

The technical application of "methanogenesis" in a bio-reactor (or digester) is designated as "biomethanation". The term "anaerobic digestion" is widely used synonymously, although it may lead to confusion with other anaerobic digestion processes that do not stringently involve generation of methane.

Practically, biomethanation has been proved a versatile, multi – purpose biological process since long time ago. It is of technical interest because it not only stabilises waste, but simultaneously acts as a net energy producer. It controls odors, reduces pathogens, minimises environmental impacts from waste emissions and it maximises resource recovery by releasing digestate as a valuable fertiliser by-product. Many of these

P. Ranalli (ed.), Improvement of Crop Plants for Industrial End Uses, 335–416.

benefits contribute directly to practical and economic factors that lead to long-term sustainable development.

The formation of gas during the decomposition of plants or animal material was first described by Robert Boyle and Denis Papin in 1682. A century after it, the release of flammable gas in nature was shown by Allessandro Volta in 1776. But it was over 100 years later, that the phenomenon was proofed to be caused by microorganisms and to be gradually exploited technologically.

First practical applications were realised at the end of the 19th century in France, United Kingdom, USA and Germany (McCarty 1982). Simple open and closed tanks (e.g. Septic tank, Talbot tank, Imhoff tank) were used for the reduction of organic matter content in sewage sludge and wastewater. Waste management requirements continued to promote the fast development of more sophisticated sewage sludge digestion- and energy recovery techniques from biogas. Tens of thousands sewage sludge digesters were built worldwide in the past decades. In 1984 the energy potential from domestic waste in the European Union was estimated to be equivalent to 10 million tons of oil (Demuynck et al. 1984).

Tremendous amounts of domestic and industrial solid waste are disposed worldwide in huge landfills, with frequent capacities of several millions of tonnes each. Several thousands of cubic meters biogas per hour are produced by natural fermentation in these landfills over long periods of 10-20 years time. Without extraction, the landfill gas escapes to the atmosphere, presenting a fire and explosion hazard, a source of damage to nearby vegetation and a substantial contribution to the greenhouse effect. For environmental and economic reasons, landfill gas extraction started in the United States in the early 1970s and spread in Europe, mainly in the United Kingdom and Germany. Meanwhile several thousand landfill gas extraction plants are in operation worldwide. Compared to all other sources of biogas, landfill gas still represents the biggest share in many countries. In 2004, for instance, 38% of the 3.2 million tons oil equivalent derived from biogas in the EU, originated from landfill gas collection (Anonym 2004).

Based on sewage sludge digester technology, first applications for industrial, anaerobic wastewater treatment were initiated during the eighties of the last century. Thousands of industrial digesters meanwhile have been successfully implemented in the food-, beverage-, sugar-, starch-, dairy-, fruit- & vegetable-, olive- & palm oil industries, slaughterhouses and other industries. Even pulp & paper-, biochemical-, pharmaceutical-, tanning-, textile- and petrochemical industry wastes are pre-treated in anaerobic digesters. By this means organic wastes have become classical feedstocks for biomethanation.

Early agricultural applications of methane fermentation aimed at simultaneous energy recovery, odor reduction and nutrient recycle. With the exception of millions of simple rural digester applications in China and India (Nyns 1986), due to its poor economics, only limited agricultural dissemination of the "1st generation" technology in the USA and Europe occurred. In the 1970s and 1980s just several hundreds of anaerobic digester applications for pig-, cattle- and chicken manure were built in Germany, Italy, Denmark, Switzerland, Austria and some other European countries (Demuynck et al. 1984). Later on, through the use of co-substrates (i.e. biogenic wastes, fat scraper contents, industrial slurries), the biogas yield and the economy of agricultural digesters could be substantially improved. Consequently, many medium- and large scale agriculture related co-digestion plants were installed in the 1990s throughout Europe. Agricultural co-digestion plants are even using source separated municipal biowastes, with subsequent recycling of the digestate to agricultural land.

In the past two decades, a new generation of commercially successful digesters were introduced for the treatment of source separated municipal bio-waste. Typical installations process 15,000 up to more than 50,000 (100,000) tons biogenic waste per year. Numerous digester types (e.g. dry- or liquid digestion) have been introduced for the use of garden- & yard waste, kitchen- & restaurant waste, market- & food waste and other biogenic waste materials. It has been estimated that in Europe, nearly 4 million tons of bio-waste will be treated annually by end of 2006 (De Baere 2005). About 124 digesters, with an average capacity between 15,000-30,000 tons per year, will be in operation.

In the last time, new driving forces for the implementation of biomethanation technology emerged. A stepwise ban for landfill of organic waste made huge amounts of organic wastes available for further treatment. Generally stricter environmental regulations for the prevention of greenhouse gas emissions, in the EU and many other countries, made biological waste treatment feasible on a broader basis. Furthermore the EU animal by-product regulation 1774/2002 approved biomethanation admissible for the treatment and hygienisation of numerous animal by-products, e.g. manure, rumen- and gut contents, other slaughterhouse wastes or food leftovers. By this means, the number of digestion plants further increased considerably during the past years.

Furthermore, a dramatic increase of fossil energy costs made biogas a more attractive, alternative renewable energy source. Additional national support programs and subsidies for bioenergy were successfully promoting the biogas application in several European countries, e.g. Sweden, Germany, Austria, Netherlands, Switzerland. Consequently biomethanation of "Energy crops", with the main goal of power supply, started dynamically. The number of energy crop digestion plants for instance doubled in Germany

within 2 years from 1,500 (2002) to more than 3,000 (2004) applications (Weiland 2004). In Denmark or Sweden, typically centralised co-digestion plants, based on manure, industrial wastes and energy crops, now produce considerable amounts of biogas which is frequently upgraded to fuel and used for public transport. It is estimated that biogas will contribute 6% to the transport fuel requirement in Sweden in 2010 (Jönsson 2005). Recent EU energy policy generally favours bioenergy as one possible alternative to fossil fuels. Huge areas of arable land and grass land will be required in the future to fulfil the challenging requirements for biomass based raw material production processes, e.g. food, animal feed, energy, raw materials for chemical and biochemical industry. According to the EU "Biomass Action Plan" (Anonym 2005a), the energetic biomass consumption should be more than doubled from 4% in 2003 (according to 69 mtoe) to 10% share of biomass (according to 185 mtoe) in 2010. By 2030, a further increase of energetic biomass use, up to 316 mtoe (according to 18% biomass share in the EU) should be possible, without significantly affecting domestic food production or complying with good agricultural practice of sustainable biomass production.

Biomethanation, with its intrinsically multifunctional character, fits well into alternative renewable energy concepts. By-products and wastes still will be a main source of substrates in the near future, but various types of energy crops are considered to be of gradually increasing importance in the future. By this means biogas will contribute a noteworthy part to the future renewable energy supply from biomass. Without doubt, widening of bioenergy will create new possibilities of rural development and employment.

2. Fundamentals of methanogenesis

2.1 The biogeochemical carbon cycle

It is estimated that about $1.55 \cdot 10^{11}$ t per year of biomass are synthesised through the photosynthesis of green plants (Kaltschmitt and Hartmann 2001). Under anaerobic conditions, the carbon previously fixed through photosysnthesis, is converted to methane by microbiological processes. Natural methane formation under presence of degradable organic material, occurs in many anaerobic habitats, e.g. in sediments of oceans, lakes, rivers, swamps, marshes, or during cellulose degradation through termites and in animal intestinal tracts. Additional, anthropogenic biogas is produced through human activities, in landfills, paddy fields and ruminant husbandry (Figure 13.1). Furthermore, methane can be partly stored as methane hydrate, under high pressure conditions of deep sea trenches.

Biological methanogenesis has been reported at temperatures ranging from 2°C (in marine sediments) to over 100°C in geothermal areas.

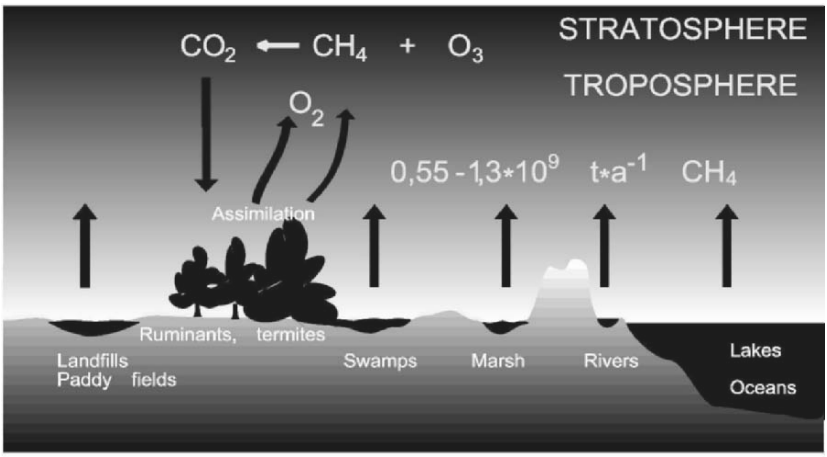


Fig. 13.1. Sources of major development and bio-geochemical cycle of methane in nature.

It has been estimated, that about 0.55-1.3 billion tons of CH_4 is released annually to the atmosphere (Koyama 1963; Vogels 1979). Recently published experimental data show (Keppler et al. 2006), that even under aerobic conditions, living and dead plant material releases considerable amounts of methane to the atmosphere. The authors estimate that about 150 million tons of CH_4 is annually released from aerobic habitats. Methane released from the various habitats, is partly reoxidised to CO_2 by methane oxidising, aerobic bacteria. But most of the methane released, reaches the atmosphere, where the CH_4 content has almost tripled since pre-industrial times and was estimated to be 1.4 ppm (Ehhalt 1974). Methane reaching the stratosphere has a mean life time of 1.5-7 years, before it is reoxidised to CO_2, through reaction products of the ozone cycle. Reoxidised CO_2 again underlies assimilation by green plants and hence closing the biogeochemical carbon cycle.

Compared to CO_2, the greenhouse effect of CH_4 is roughly 27 times higher. Therefore anthropogenic emissions of methane must be prevented at all stages, respectively a proper use of generated CH_4 (i.e. combustion to CO_2) must be assured.

2.2 Microbiology of methane formation

2.2.1 Stepwise decomposition of substrates

Although the formation of combustible gas in the nature was already known since 1776, it was not before the end of the 19th century, that the gas formation was associated to the activity of micro organisms (Bechamp 1868). Early investigations, describing the biological biogas formation as a one step, single species process (Figure 13.2), were carried out by Omelianski (1906). After this, a more detailed reaction scheme for the methane fermentation, divided the process into two phases, an “acidification” or "acid" phase and a “methanogenic” or "methane" phase. This scheme described by Barker (1956), showed already the principal components of the complex anaerobic metabolisation of carbohydrates, proteins and lipids. Subsequent studies proved a multistep character of the methane formation (Bryant 1974). The multiphase nature of the process was further on revealed in detail by the discovery of hydrogen-producing acetogenic bacteria (McInerney and Bryant 1980) and by a better appreciation of the limited substrate capabilities of methanogenic bacteria (Bryant 1977).

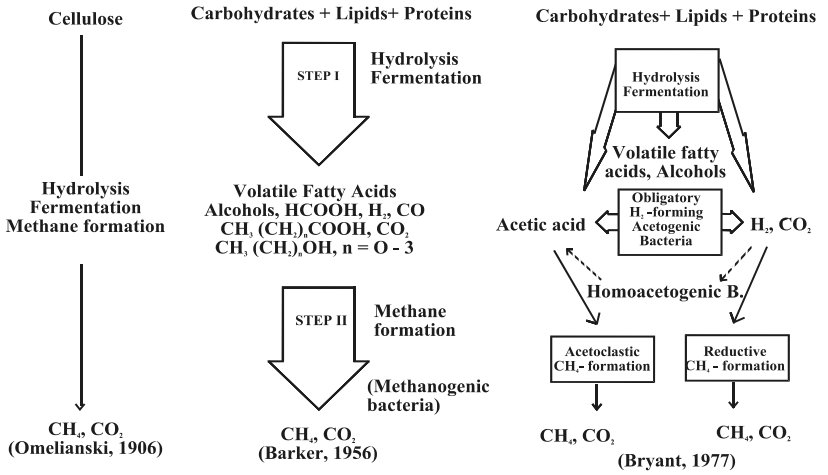


Fig. 13.2. Development of the methanogenesis pattern from a one step (1906) and two step (1956), to the current three step model (1977).

Currently methanogenesis is agreed to consist of a series of reactions, which are catalysed by mixed groups of bacterial species, through which organic matter is converted in a stepwise reaction to the main products methane and carbon dioxide (Figure 13.2). Three major groups of

microorganisms have been identified with specific functions in the overall process (Schink 1992). Proteins, lipids and carbohydrates are initially hydrolysed to oligomers or monomers, which are then metabolised by fermentative bacteria with the production of hydrogen (H_2), carbon dioxide (CO_2), and volatile organic acids such as acetate, propionate and butyrate. The volatile organic acids, other than acetate, are converted to methanogenic precursors (H_2, CO_2 and acetate) by a group of bacteria named syntrophic acetogens. Finally, the methanogenic bacteria produce methane (CH_4) from simple molecules i.e. acetate, formiate, methanol, methanolamin or from H_2 and CO_2. Almost all known methanogenic bacteria convert H_2/CO_2 to methane, whilst aceticlastic methanogenesis has been documented for only two methanogenic genera i.e. *Methanosarcina* and *Methanosaeta (Methanothrix*). Roughly 70% of the methane is formed from acetate, the remaining 30% is built by reduction of CO_2 through H_2 (Mah 1977).

All steps of the serial metabolisation, i.e. hydrolysis, acidogenesis and methane formation are rate-controlled by the slowest member involved in the respective process. Therefore accumulation of inhibiting metabolites may occur. If this form of substrate is a non-acid organic (e.g. alcohol, solvent), there may be no adverse impact on the overall consortia. But the slowest members of the consortia often are the propionic- or acetic acid - utilisers, so that an accumulation of these organic acids can overwhelm the available bicarbonate alkalinity. Such a malfunction may cause a drop in pH which can have a drastically adverse impact upon the entire microbial consortia. Unfortunately the greatest inhibition of a low pH may be directed at the propionic- and acetic acid - utilisers themselves.

It is evident from this series reaction, that the anaerobic process works reliable, as long as each subsequent class of organisms processes the organic intermediates at least as fast as they are produced. Since microbial processes function at a rate proportional to their substrate concentration, an accumulation of substrate may result before they are able to process it as fast as it is passed on to them.

For achieving a stable fermentation process, a proper balanced nutrient composition must be available and the involved bacterial species must interact in a dynamic equilibrium. Since some of the intermediate metabolites (hydrogen, propionate, ammonia, sulphide) can become inhibitory, metabolite concentrations must be kept fairly low and, by controlled feeding, the pH of the system must be kept near neutral. Maintenance of a low hydrogen partial pressure, which is primarily dependent upon the activity of the hydrogen-utilizing methanogens, regulates the degradation of propionate and butyrate. The acetate-utilizing methanogens regulate the pH by conversion of acetic acid to methane and CO_2. For thermodynamic reasons, the latter transformation can only take place at very low partial pressures

of hydrogen. In stable, efficient methanogenesis, the hydrogen partial pressure ranges between 1 and 10 Pa. An abrupt increase in hydrogen partial pressure or a hydrogen partial pressure above 20 Pa is indicative of malfunction. The increased partial pressure of hydrogen results in accumulation of intermediate organic metabolites, with subsequent pH drop below 6, finally ceasing methane generation. The adverse effect of increased volatile fatty acid concentrations on methane bacteria has been extensively investigated. Especially propionate was inhibiting pure cultures of *Methanobacterium formicicum*, even at concentrations of 1 $g.l^{-1}$ sodiumpropionate, while up to 10 $g.l^{-1}$ sodiumacetate and –butyrate were tolerated (Shaw 1971). Upon adaptation, up to 6 $g.l^{-1}$ propionate were tolerated in mixed culture continuous laboratory fermentations of molasses slops (Braun and Huss 1982). Increased propionate concentrations have been frequently reported from practical mixed culture digestion processes (Pohland and Bloodgood 1963; Andrews 1969). In most cases increasing propionate signalised inconsistencies in the overall reaction equilibrium. Usually the overall fatty acid content in methanogenesis remains far below 1 $g.l^{-1}$.

2.2.2 Microbial interactions and competition

During methanogenesis numerous acid-forming bacteria are associated with methanogens. The products of fermentation vary considerably, depending on the bacteria involved in the fermentation. Therefore, changes in environmental conditions that result in changes in dominant bacterial species, also result in changes in the concentrations of acids, alcohols etc. that are produced during fermentation. Such changes determine the substrates ultimately available for the methanogens, their activity and, consequently, overall performance of methanogenesis.

Dependent on the substrate composition, a competition for the available hydrogen can occur during methanogenesis. In the presence of oxidised nitrogen (e.g. NO_3^-) or sulphur (e.g. SO_4^{2-}), non - methanogenic bacteria may interfere with hydrogen transfer to CO_2. Acetogenic bacteria can reduce inorganic carbon with hydrogen to produce acetate and sulfate-reducing bacteria (e.g. *Desulfovibrio desulfuricans*) reduce sulfate with hydrogen to form hydrogen sulphide:

$$H^+ + SO_4^{2-} + 4\ H_2 = HS^- + 4\ H_2O \qquad \Delta G_O` = -\ 152\ kJ/mol$$

When sulfate-reducing bacteria and methane-producing bacteria compete for hydrogen and acetate, for thermodynamic reasons, sulfate-reducing bacteria obtain hydrogen and acetate more easily than methane-forming bacteria under low-acetate concentrations. With high sulfur containing substrates, usually high H_2S – contents result during methanogenesis. Depending on the fermentation temperature and pH, increasing free sulfide levels may inhibit all reaction stages. Depending on environmental conditions and adaptation, total H_2S levels up to 100-200 $mg.l^{-1}$ may be tolerable (Lawrence and McCarty 1964; Hobson et al. 1981; Pfeffer 1979). Total H_2S – levels higher than 300 $mg.l^{-1}$ or free H_2S concentrations higher than 150 $mg.l^{-1}$ cause severe inhibition of all bacteria involved. Due to precipitation, presence of heavy metals may counteract sulfide toxicity and may allow higher H_2S concentrations in substrates. Sulfide toxicity is reversible and bacteria may gradually recover after H_2S reduction.

Ammonia is typically formed during methanogenesis through the proteolytic activity of hydrolytic- and fermentative bacteria. Proteolysis and subsequently deamination of amino acids (equation 13.1) may cause high NH_3 levels, with potential inhibitory effects. The deamination of amino acids results in the production of a variety of organic acids including acetate and butyrate. Ammonia is released during the degradation of amino acids. Acetate and butyrate serve as substrates for methanogens, whereas ammonia increases the digester alkalinity or may contribute to ammonia toxicity in all fermentation stages.

Furthermore NH_3 can be formed through reduction of nitrate (equation 13.2). As do sulfate reducers, also nitrate reducing bacteria compete successfully for the hydrogen normally used for CO_2 reduction to methane. Substrates with high protein- or nitrate contents may therefore cause less CH_4-formation and considerable ammonia toxicity during methanogenesis. With increasing temperature and pH, the concentration of free NH_3 increases (Equation 13.3) and can cause complete process inhibition. Although bacteria can well be adopted to high NH_3 levels, depending on substrate composition, pH and temperature, increased concentrations frequently are reason for malfunction of biomethanation processes. A concentration of 1,500-3,000 $mg.l^{-1}$ NH_4^+ has been reported inhibitory by McCarty and McKinney (1961). Other reports show tolerance of gradually adopted methanogenesis to NH_4^+ concentrations of 5,000 $mg.l^{-1}$ (Van Velsen 1979; Braun et al. 1981).

$$4\ H_2NCH_2COOH + 2\ H_2O = 4\ NH_3 + 2\ CO_2 + 3\ CH_3COOH \qquad (13.1)$$

$$NO_3^- + H^+ + 4\,H_2 = NH_4^+ + OH^- + 2\,H_2O \quad (13.2)$$

$$NH_3 + H_2O = NH_4^+ + OH^- \quad (13.3)$$

3. Biomethanation technology

3.1 Process engineering

From the process engineering point of view, biomethanation is a relatively simple reaction. Since the process uses a "mixed culture" of ubiquitous organisms, usually no substrate sterilization steps or further measures for culture enrichment, -maintenance or preconditioning are required. A product separation step is unnecessary, as the biogas separates itself from the aqueous phase. Since the methane produced is fairly insoluble in water, it does not accumulate to inhibitory concentrations in the fermentation liquor. Under favourable conditions, the second product from biomethanation, i.e. fermentation residue (digestate) can be spread as fertiliser on agricultural land. If a direct use of the digestate is not possible, further conditioning, e.g. separation of solids and subsequent composting or drying, with following post-treatment of the liquid digestate are required.

A generalised, simplified scheme of the technical biomethanation process (Figure 13.3) comprises the 4 steps substrate delivery, pre-treatment and storage (1), digestion (2), digestate use (3) and energy recovery from biogas (4). Depending on the substrates applied (e.g. sewage sludge, biowaste, industrial by-products, energy crops), the delivered material frequently must undergo extensive pre-treatment steps. By means of sieving, wind sorting, cyclones, magnetic belts, contaminants like sand, cullets, metals, rubber, plastics etc. have to be properly sorted out.

Depending on the digester system applied, under optimum conditions, the purified waste stream is continuously fed into the digester. Various dosage systems for liquid- (i.e. rotary pumps, displacement pumps) and solid wastes (i.e. high pressure piston pumps, auger feeding) have been developed for the dosage of highly variable consistency wastes.

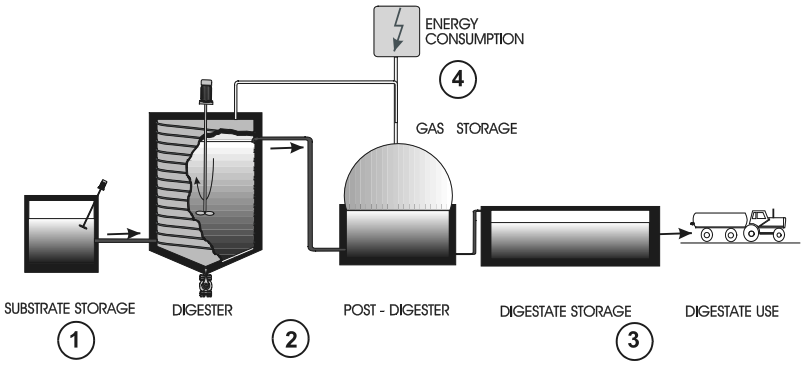


Fig. 13.3. General schematic drawing of a typical AD application, comprising substrate preparation and –storage (1), digestion (2), storage and use of digestate (3) and storage-, purification- and upgrading of the produced biogas.

Usually the effluent leaves the digester by gravity flow and in most cases undergoes further digestion in a second "post digester" step. Digester and post-digester have thermal insulations, while a mixing equipment in most cases is only installed in the digester step.

The final digestate storage tank is designed for storing periods of many months and usually does not include thermal insulation. Most conventional storage tanks also did not include coverings. Since methane formation at very reduced rates is still continuing at ambient storage temperatures, for environmental protection reasons, newly built plants, in most cases include storage tank coverings. The head space of the digestate storage tank must be connected to the gas collection, respectively gas security system.

In most conventional agricultural applications of biogas plants (i.e. manure digestion, co-digestion of biogenic wastes), the digestate after respective storage periods, is directly applied as fertiliser and soil conditioner in crop production or on pasture land. With increasing plant size (e.g. energy crop digestion plants) and lack of nearby agricultural areas, further treatment of the digestate frequently is required. In most cases dewatering of the digestate with subsequent composting of the solids and use of the liquid fraction as fertiliser is applied. If the liquid fraction cannot be used directly, further upgrading (i.e. NH_3 – recovery, phosphate removal) can be necessary.

Released biogas is collected in both digestion steps and stored in gas storage tanks or, most frequently in the head space of post digesters, covered with floating, gas tight membranes. Depending on its final use, biogas must undergo several purification steps. The vast majority of agricultural

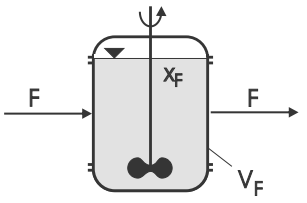
digesters applies a simultaneous desulphurisation by controlled addition of air into the digesters head space. By this means gas qualities required for combustion in burners or CHP engines can be met. If biogas is intended for the use as transport fuel or to be fed into the natural gas grid, further upgrading, i.e. CO_2 removal, has to be implemented for purification.

3.2 Process start up

For the start up normally no specific commercial seeding material is available and no special measures for pure culture fermentation are applied. The required course of fermentation therefore must be controlled by the cultivation conditions, i.e. temperature, substrate composition, sludge loading rate and hydraulic residence time during start up. If seeding material, e.g. digester sludge from comparable processes or digested sewage sludge is available, the start up process can be easily initiated by adding seed sludge (20-30% v/v), together with small amounts of substrate into the digester. If no convenient seed sludge is available in sufficient amount, the digester must be filled with water and small amounts of diluted substrate. The sludge loading rate (g VS per g bacterial sludge and day) must be carefully increased in small steps during process start up, enabling sufficient growth and increase of bacteria. The corresponding hydraulic residence time (days) must be high enough to allow a complete use of the substrate. By regular control of substrate degradation respectively biogas formation, a gradual increase of sludge loading can be enabled. The substrate dosing rate must only be increased when the corresponding biogas yield and substrate degradation have been realised. The start up process may take several weeks or months and is crucial for the establishment of a proper mixed bacterial culture and the achievement of a stable methanogenesis and substrate degradation. According to Summers and Bousfield (1980), following an inoculation with digested sewage sludge, a stable equilibrium can be expected in swine manure digestion after 5 months start up operation. When adopted seeding materials from comparable digesters are available, the start up procedure can be much faster. By controlling gas formation and substrate addition, in a full scale energy crop (maize silage) digestion, the maximum loading rate could be achieved within 6 weeks after inoculation with cow manure (Resch 2005).

3.3 Operational parameters

As a common measure in continuous reactor operation, usually the daily substrate feeding rate, or flow rate (F) is broadly applied (Figure 13.4). Usually the substrate is dosed in a semi-continuous or preferably, in



F	($l.d^{-1}$)	Substrate flow rate
X_F	(g)	Bacteria content
V_F	(l)	Reactor volume
θ	(d)	Hydraulic residence time
S	($g.d^{-1}$)	Substrate dosage
B_{TS}	($g.g^{-1}.d^{-1}$)	Sludge loading
B_V	($g.l^{-1}.d^{-1}$)	Volumetric loading

$$\theta = \frac{V_F}{F} \qquad B_{TS} = \frac{S}{X_F} \qquad B_V = \frac{S}{V_F}$$

Fig. 13.4. Schematic sketch of the one step continuous reactor system and characterising operational parameters.

continuous mode to the digester. In case of semi-continuous dosing substrate is added at least once a day. Preferably semi-continuous dosing should be performed several times (6-12) per day in order to keep constant growth conditions for the bacterial population and to achieve a constant biogas delivery.

If the flow rate is referred to the active reactor volume (V_F) the mean residence time θ (days) of the substrate in the reaction vessel can be calculated. Under practical conditions, residence times cover a broad range, from few hours in case of easy degradable soluble wastes, up to several months (e.g. 80-100 days) for example in case of energy crop digestion. Long term measurements of full scale biogas plants in Germany showed a range of 30-120 days residence time (Weiland 2004), while similar Austrian investigations resulted in a median value of 133 days hydraulic residence time (Laaber et al. 2005).

In addition to the mean residence time, the turnover rate can be more precisely defined by using the term loading. The loading rate defines the amount of substrate being fed to the digester within a defined time span. If the substrate loading rate is referred to the reactor volume (see Fig. 13.4) it is designated volumetric loading rate (B_V). If reference is made to the available bacterial sludge content in the digester (X_F) it is commonly called sludge loading (B_{TS}). Both measures allow the comparison of different reactor systems and can be used for process dimensioning and –control.

Under practical conditions the possible maximum loading rate is dependent on the concentration of viable biomass which can be retained in the reactor. Furthermore substrate properties (e.g. concentration, degradability), environmental conditions (e.g. temperature, pH) and reactor performance (e.g. configuration, mass transfer capability, scum- and bottom layer formation etc.) determine the maximum allowable loading rate. Depending on the influential factors listed, volumetric loadings for the same substrate can vary in the range between 5-40 $g.l^{-1}.d^{-1}$ VS (Braun 1982).

With increasing loading, the biogas yield and the degradation efficiency can be adversely affected. For practical reasons (biogas yield) and to achieve the environmental requirements of emissions avoidance, optimum loading rates and residence times for maximum substrate degradation should be envisaged. Good indicators for reactor overloading are a decline of the methane content in biogas, the increase of the volatile fatty acid content in the digestate and variations of the pH value.

From practical experience in bioreactor monitoring with numerous substrates, limiting values of operational parameters can be derived for good biogas practice in digester operation. Since residence time as well as loading rates are case specific parameters, preferably metabolite concentrations should be considered for process control. An acceptable range for the pH is 7-8, for volatile fatty acids between 1,500 -4,500 $mg.\ l^{-1}$ and the NH_4^+-concentration should not exceed 5,000 $mg.l^{-1}$. A pH value below 7 or above 8, fatty acid contents exceeding 4,500 $mg.l^{-1}$ and NH_4^+-concentrations above 4,500 $mg.l^{-1}$ are clear indications for increasing inconsistency. Keeping within the optimum ranges usually guarantees reliable and good process performance, maximum biogas yields and VS degradation. Continuing deviations from the optimum operational parameter ranges should be considered serious, since recovery periods after digester failures can take several weeks or months and in some cases can require re-inoculation and restart of the process. Most frequently overloading, or the use of unknown substrates, are the main reasons for process failures. Keeping within the predefined case specific loading rates and residence time, usually guarantees a stable process performance. Nevertheless for reliable operation a frequent measurement of metabolite concentrations and of the methane content in the produced biogas is highly recommendable.

3.4 Process control

The biochemical processes involved in methanogenesis are complex and much efforts were undertaken to enhancing the process stability and introducing proper process control measures. Nevertheless, the degree of automation of the technical process is still low and the fermentation requires

comprehensive manual control and monitoring for reliable and save operation.

Minimum requirements for a proper process control include continuous measurement of temperature, pH, biogas production and methane content. Furthermore type and mass of the substrate fed to the digester must be recorded on a daily basis. Depending on the process stability achieved, TS-, VS- and volatile fatty acid contents in the digester should be measured on a regular basis. In case of changing substrates, loading rates or obvious process instabilities, the minimum frequency of measurements should be once a week. During stable fermentation periods monitoring can be fairly extended to several weeks or months. The measurement of temperature, pH, biogas production, methane- and H_2S-content can be fully automated and measured on-line with commercially available standard equipment. For the measurement of TS, VS and volatile fatty acid metabolites, standard methods are available (DEV, 2005; APHA, 1998). For the detection of volatile fatty acids numerous titration-, chromatographic- and spectrometric methods have been proposed (Kolb 1986; Lindorfer 2003; Boe et al. 2005).

For environmental protection reasons the emissions from biogas combustion must be controlled by a proper regulation of burners or engines. Furthermore the hygienic status of the digestate must be controlled. Depending on the digested waste and the intended final use of the digestate in agriculture, composting or landscape architecture, different requirements of hygienisation have to be fulfilled. In case of animal by-product digestion the European regulation on animal by-products (EC) 1774/2002 has to be followed.

3.5 Substrate composition

For stable fermentation and optimisation of biomethanation, among other conditions, a proper balanced nutrient composition must be provided in the substrate. The main nutrients, carbon, nitrogen, phosphorous and sulfur, must be available in a ratio according to the empirical formula $C_5H_7O_2NP_{0.06}S_{0.1}$ of anaerobically grown cell biomass. Lack of nutrients or improper balanced nutrients can considerably retard formation-, disturb fermentation and reduce the yield of methane. An optimum C : N -ratio between 25-32 is considered to give best performance (Angelidaki et al. 2003). The C : N ratio in natural substrates can vary considerably (Table 13.1) and ratios of less than 16 : 1 (Sanders and Bloodgood 1965; Braun 1982) or higher than 45 : 1 (De Renzo 1977) may occur. Typical substrates with surplus of nitrogen origin from slaughterhouses or rendering plants,

Table 13.1. C : N ratio, water content and content of organic substance in various substrates commonly applied for biomethanation.

	C : N-ratio	Water content (%)	VS (% of TS)
Wood waste	723	30-40	99.6
Sawdust	511	20-80	95
Wood chips	60-150	10-30	95
Straw	90	30	90
Bark	20-500	50	90
Paper	173	4-16	74
Municipal waste	15-18	50-60	30-40
Market waste	10-20	90	83
Kitchen & restaurant waste	10-20	75-85	80-90
Fat trap contents	50-200	30-75	80-96
Stomach- & gut contents	11-21	87-89	85
Fish waste	3-4	75-80	70-85
Leaves (green)	40-80	20	90
Leaves (wilted)	30-60	20	90
Fruit waste	35	80-85	75
Grass cuttings	12-25	75-80	90
Waste mycelium	19	75-90	70-90
Yeast waste	3	80-90	80
Digested sludge	15.7	92-96	50-60
Sewage sludge	6.3	95.5-99.5	50-60
Chicken manure	7-11	40-60	60

while lack of nitrogen occurs in pulp and paper wastes or some plant materials (e.g. straw, saw dust). In case of higher variations, correction measures have to be undertaken since lack of nitrogen retards growth of bacteria, while a surplus can rapidly cause ammonia toxicity. For practical purposes a COD:N:P ratio of 800 : 5 : 1 has been proposed as minimum requirement in biomethanation (Böhnke et al. 1993).

Most practically applied substrates do not require any supplements. The demand of phosphorous and sulphur can easily be satisfied by most common substrates. In few cases of technical applications (e.g. exhaust vapors), a lack of nutrients and essential trace elements can derogate the reaction performance. Furthermore, in some industrial wastes, the bioavailability of trace elements can be insufficient, even though the required elements are physically present in the substrate. Due to their very strong binding properties, chelated forms of trace metals can be reasons for an insufficient bioavailability. Furthermore precipitations as metal sulfides frequently occur in high sulfur containing wastes.

3.6 Inhibiting substrate components

Numerous inhibiting substrate components can cause a malfunction of methanogenesis. Similar to the effects of adverse environmental conditions (e.g. pH drop, temperature change, metabolite accumulations), inhibitory or toxic compounds can cause retarded methane formation, a decrease of the methane content in biogas or can even cause complete failure of methanogenesis. Possible inhibition has been reported from long chain fatty acids such as oleate and stearate, from some antibiotics, from phenols, chloroform and higher levels (10^{-3} to 10^{-4} Mol) of heavy metals (Mosey et al. 1971; McBride and Wolfe 1971; Sykes and Kirsch 1972; Henderson 1973). In most cases the inhibition is reversible and disappears after removal of the toxicant. Furthermore gradual adaptation to increased levels of inhibitory substances can be frequently observed and even complete degradation of toxic compounds, e.g. pentachlorphenol, or detoxification of nitroaromatic compounds, chlorinated aliphatic or aromatic hydrocarbons and azo dyes occurs.

An adverse effect of inhibiting substrate components usually can be detected through fermentation tests with different dilutions of substrate samples.

3.7 Substrate pre-treatment

Through sufficient pre-treatment and removal of contaminants, an interference or obstruction of the methanogenesis process in the digester must be safely avoided. Once problems already appear within the digester (e.g. scum formation, foaming, bottom layers), solutions are very hard to realise in most cases. As a principal requirement, a homogeneous mixture of the digester contents must be ensured at any time, allowing an undisturbed mass transfer between solid particles, liquid and gas phase. The incoming substrate must be homogeneously distributed within the digestion vessel and the gas bubbles formed should be immediately released from the cells or aggregates. In dependence on the reactor system applied (e.g. mixing capacity, prevention of phase separation) and the substrates composition and physical state (e.g. fluiddynamic properties, settling characteristics), fairly different degrees of substrate pre-treatment can be required in the various biomethanation processes.

In some cases a high dry matter content of substrates to some extent can require substrate dilution with water or with other, less concentrated substrates. Depending on the fluid dynamic properties of the substrate (i.e. viscosity, particle size distribution), homogeneous, liquid phase digester

systems, usually demand total solids contents of less than 10% TS. Dry fermentation systems (solid state fermentation) operate at TS concentrations of about 20-35%.

Some substrates used in practice for biomethanation, to a different extent require extended pre-treatment. As described above, the most simple pre-treatment is substrate equalisation by dilution. By this means, ditrimental influences of pH variations, too high concentrations or inhibiting effects of substrates can often be avoided. Additionally, many practical substrates demand homogenisation and crushing of coarse and bulky material. By this means a faster biodegradation can be achieved and clogging of the piping system through coarse particles can be avoided. Furthermore the scum formation, the formation of bottom layers and reduction of the effective reactor volume can be prevented, or at least considerably retarded.

If excessive amounts of inert contaminants are contained in substrates (e.g. source separated municipal biowaste), an extended pre-separation step for the removal of disturbing materials has to implemented. Typical contaminants to be removed from such wastes are sand, gravel, cullets, metals, plastic, rubber or compound materials. These type of substrates can require sophisticated, comprehensive and costly pre-treatment equipment.

The European regulation (EC) 1774/2002 defines allowable animal by-product substrates (e.g. manure, stomach contents, restaurant- & kitchen waste, food waste) and defines treatment conditions for the use in biomethanation. Some substrates (e.g. animal by-products) can even require thermal sterilisation (133^{o}C) or pasteurisation (70^{o}C) for safety reasons.

Energy crop digestion plants usually implement a substrate (crop) ensilage step for the conservation and storage of the substrate. Fresh crop material is chopped, compacted in silos and covered airtight. The pH reduction (about pH 4), performed through an autochthonic mixed population of lactic acid bacteria and yeasts, stabilises the plant material for the continuous use in biomethanation over the year.

3.8 Mixing of reactor contents

As stated earlier, together with sufficient substrate pre-treatment, proper mixing of the reactor contents has to ensure a reliable mass transfer between solid particles, liquid and gas phase of the substrate. In contrast to aerobic (aerated) bioreactors, the energy input in anaerobic fermentation systems may be limited to the requirements of homogenisation. Inadequate (Finney and Evans 1975), as well as exorbitant mixing (Stafford et al. 1980) have been reported as reasons for retarded methanogenesis. Insufficient mixing cannot efficiently remove gas coatings from cell aggregates, while heavy turbulence may disturb the fragile equilibrium between the

different active bacterial species. Furthermore gas bubbles captured in cell aggregates generate a lifting tendency and may cause compact scum layers followed by severe operational- and safety problems. Generalised optimum mixing capacities cannot be predefined. However, from practical experience a minimum mixing requirement has to safely prevent scum layers and sediment formation.

Principally mixing of the reactor contents can be achieved by mechanical equipment (e.g. stirrers), hydraulic measures (e.g. turbines, pumps) or by means of gas injection into the reactor (Figure 13.5). The choice of a suitable mixing equipment depends on the present substrate and reaction type. The stirred vessel or stirred tank reactor, is still the most widely applied bioreactor for biomethanation. It consists of a cylindrical vessel with a diameter to height ratio of about 1 : 3. All 3 types of mixing can be alternatively implemented into a stirred tank reactor. Even combinations of systems, e.g. mechanical mixer (propeller), supported by circulating pumps, or gas injection supported by hydraulic mixing through biogas displacement (Braun 1982) have been realised. The minimum gas volumetric rate for sufficient mixing in cylindrical vessels has been defined with 6 $m^3.m^{-2}.h^{-1}$ (Hobson et al. 1981). Morgan and Neuspiel (1958) have elucidated the calculation formula $Q = k.v^3.D$ ($m^3.min^{-1}$). For cylindrical vessels with the diameter D, k is between 10-13 and a gas velocity of 11 $m.min^{-1}$ is considered to be sufficient. Gas mixing represents the most economic technique, but can be problematic through foam- and scum layer formation with some substrates (e.g. rumen contents, straw).

Depending on the substrate viscosity and particle size distribution, stirred tank reactors, or so called “wet digestion systems”, normally can scope with TS contents up to about 8-12 (15)%. With increasing viscosities, respectively TS contents, channel formation may occur and homogeneous conditions cannot be ensured. When applied in stirred tank reactors, most substrates with TS contents exceeding 12-15% require dilution or pre-treatment (e.g. Separation of particulate matter, hydrolysis).

Dry fermentation systems have been developed to scope with elevated TS contents of low water containing substrates e.g. plant materials, municipal bio-waste. Furthermore, by using undiluted substrates, wastewater accumulation during digestion can be minimised, compared to wet digestion systems. At TS contents of 20-35% highly efficient mixing devices have to be implemented to achieve a proper mass transfer, especially sufficient degassing of compact aggregates between substrate particles and bacteria. High pressure piston pumps are frequently used for mixing in vertical cylindrical vessels. Since the whole reactor content has to be circulated several times a day, considerable energy input has to be provided for sufficient mixing. In horizontal vessels, slow moving blade or screw stirrers are often applied as mixing and transport devices.

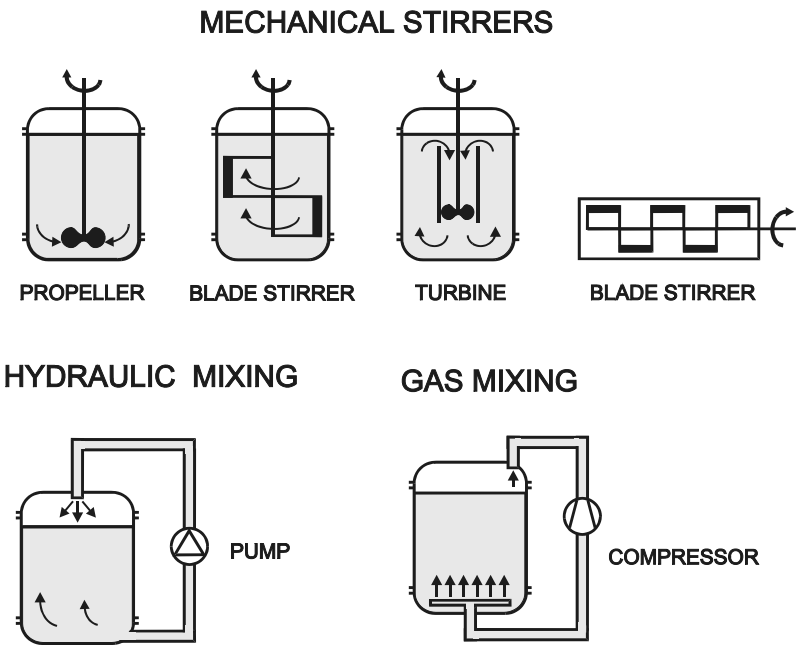


Fig. 13.5. Mechanical-, hydraulic- and gas mixing principles in bio-reactor systems.

To avoid possible problems with high TS containing substrates, special reactor combinations have been realised sporadically. Percolation systems distribute liquid effluent (digestate) from a first bioreactor step as a leaching agent over solid substrate components contained in the second percolation step. Solid material is continuously fed into the second step and soluble extract is recycled to the first bioreactor step. Other reaction systems extract the liquid fraction from solid materials by means of mechanical devices. Different high pressure squeezing machines are used to obtain high yields of soluble fractions from solid organic materials. After complete extraction, the solid residues from percolation or from press separation processes, usually are brought to composting. The extracted liquid fraction can be easily used in high rate biomethanation systems.

3.9 Temperature

Most technical digestion applications have been realised under either mesophilic (30-40°C), or thermophilic (50-60°C) temperatures, fewer applications are reported using ambient (15-25°C) temperatures. Digesters with lower temperatures (<40°C) are more stable and normally require less

process energy, but due to lower reaction rates, can need larger reactor volumes.

Not just the reaction velocity will be influenced by the reactor temperature, but also chemical dissociation, physical diffusion and, according to Henry's law, liquid - gaseous particle distribution change with alternating temperatures. Hence, toxicity of metabolites, i.e. NH_3, can be heavily increased at elevated, thermophilic temperatures. In general, methanogenesis is more sensitive to possible disturbances at an elevated, thermophilic temperature range.

If temperature changes occur very slow, bacteria usually adopt well to changing conditions. A continuous temperature increase of 1°C per week caused no break in gas formation. Faster changes can temporarily interrupt the fermentation process, subsequently demanding extended periods of recovery for regular performance.

The vast majority of technical digesters needs external heat supply for heating up the incoming substrate and to equalise the heat losses through surface radiation. The exergonic reaction heat of the microbial- and chemical reactions usually is not sufficient for stabilising the fermentation at mesophilic- or thermophilic temperatures. Nevertheless in cases of very high substrate concentrations, e.g. in energy crop digestion, a gradual increase from mesophilic to sub-thermophilic temperature has been reported (Lindorfer et al. 2005a). As a matter of fact, many of these digesters can be operated at elevated, sub-thermophilic temperatures of about 42°C without any external heat supply.

3.10 pH

The pH value of the fermentation broth plays a predominant role, interfering with almost every running reaction. Methanogens performing the final fermentation step, prefer nearly neutral pH conditions, with a generally accepted optimum pH-range from approximately 6.5-8.2. Some exceptions e.g. *Methanosarcina mazei*, are reported to even operate at slightly lower pH ranges. Values below or above the mentioned pH range usually decrease the methane production rate rather steeply. Nevertheless methane formation will continue at pH 6 and even lower, at noticeable reduced rates. At pH 5 roughly 25% of the methane production rate observed under neutral pH conditions was reported in individual cases (Speece 1996). Since the buffer capacity under reduced pH conditions usually is insufficient, fermentations tend to result in considerable instability. Cells organised in biofilms, flocs or granules, generally are less susceptible to disturbances caused from changing environments. Under such conditions,

changes in pH or temperature therefore cause less dysfunction in methane fermentations as well (Suidan et al. 1983).

Long term experiences with a large number of full scale agricultural biomethanation plants (continuously stirred tank reactor type) have shown a common pH - range between 7.4-8.1 under practical conditions (Laaber et al. 2005). Deviations of the pH from the acceptable range 6.5-8.2 are often caused by overloading of reactors. Loading reduction, or dilution of substrates, usually re-establishes a proper pH value. In exceptional cases a short term addition of lime ($Ca(OH)_2$) or preferably soda (Na_2CO_3) is required for stabilising the pH value around 7. Care has to be taken when $Ca(OH)_2$ is added for prolonged periods. Precipitations and crystallisations with calcium can cause clogging of pipings, valves, heat exchangers and therefore cause severe long term damage in technical equipment.

In some exceptional cases of easy degradable, (e.g. high sugar containing) substrates of low buffer capacity, due to very fast hydrolysis, the pH may easily drop below 6.5, causing retarded methane formation in single step reactor configurations. Such a fast pH drop may justify a 2 – step reactor configuration, separating the hydrolysis process (step 1) from the methane formation (step 2). The hydrolysis reactor (step 1) can be operated at lower pH values (i.e. 6.5-4) while the methane formation (step 2) can take place at optimum pH conditions (i.e. 6.5-8.2). By controlled dosage of substrate from the hydrolysis step to the second methanogenesis step, the required equilibrium between hydrolytic and methanogenic organisms can be sustained more reliably.

In practice, the hydrolysis process frequently already occurs in substrate storage tanks. Special care has to be taken therefore to avoid unwanted gas release during substrate storage. If delivered substrates cannot be processed „just in time“, Good Biogas Practice in such cases requires closed substrate storage tanks and implementation of possibly released gas into the gas collection, respectively –security system of the biogas plant.

3.11 Biogas yield

Provided the elemental composition of a substrate is known, the respective biogas yield and –composition can be calculated, based on the stoichiometric reaction, which following is shown for instance for glucose (equation 13.4)

$$C_6H_{12}O_6 = 3\ CH_4 + 3\ CO_2 \qquad (13.4)$$

As a result, under standard conditions (0°C, 101.325 kPa), theoretically 1 mole glucose gives 2 x 3 moles gas (50% CO_2 + 50% CH_4), or 180 g glucose result in the formation of 6 x 22.4 = 134.4 l Biogas. Similarly the

biogas yields and gas compositions for lipids and proteins can be estimated (Table 13.2).

Table 13.2. Theoretical biogas yield and –composition of carbohydrates, lipids and proteins.

Substrate	Biogas yield (l/g substrate used)	$\%CO_2$	$\%CH_4$
Carbohydrates	0.747	50	50
Lipids	1.25	32	68
Proteins	0.7	29	71

Numerous efforts have been undertaken for the development of easily manageable calculation methods, for the reliable prediction of the methane formation potential from unknown substrates. Most methods proposed, are based on the chemical composition, or on the nutrient value of the organic substrate. Although being more time consuming and elaborate, the experimental fermentation test (Figure 13.6) of unknown substrates gives reliable information on biogas (methane) yields and allows for the determination of possible influential factors on the course of fermentation. Standardised fermentation tests are available (DEV, 2005).

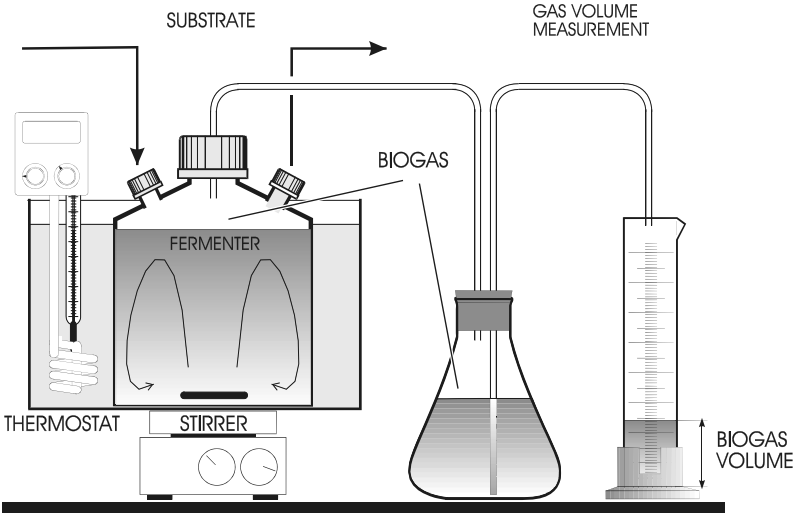


Fig. 13.6. Sketch of fermentation test equipment for the determination of the biological methane potential of organic materials. Water displacement is measured in an alkaline solution absorbing the CO_2 from the biogas evolved.

Typically the tests are run with strongly diluted substrate samples in batch-wise mode. The degree of required dilution depends on the substrate VS concentration and must safely prevent inhibition of methanogenesis through possible excessive metabolite formation, e.g. volatile fatty acids, NH_3 Usually 20-30% (v/v) standardised seeding sludge is added to the samples and the gas formation and –quality are followed during several weeks at constant temperature. Using similar equipment, early systematic investigations of biogas yields from numerous agricultural wastes, by-products and plant- or crop materials have already been early published by Reinhold and Noak (1956). A compilation of exemplary gas yields from fermentation testing in the authors laboratory is given in Table 13.3. As can be seen the biogas yields obtained varied between 0.1-0.2 (e.g. Grey waste, Sewage sludge), 0.3-0.6 (e.g. Manure, slops, industrial wastes) and 0.7-1.36 $m^3.kg^{-1}$ VS (e.g. Waste blood plasma, fat, oil). A similar broad range for the minimum required residence time, from 10 days (potato distillery slops) to 62 days (stomach-, rumen contents), was required for sufficient degradation.

Under practical conditions, the biogas yield obtained can be influenced through numerous factors. Inadequate substrate concentration and nutrient composition, inhibiting substrate components or metabolite formation and inhibiting process operational conditions e.g. improper temperature, excessive loading rate, insufficient residence time or insufficient bacteria content can adversely affect methanogenesis. For obtaining reliable values on biogas yields and reactor performance from unknown substrates, it can be necessary to perform continuous fermentation tests under controlled laboratory conditions. Based on a standardised batch-wise fermentation test, substrate is continuously added at slowly increasing dosing rates and –concentrations. The substrate dosing rate can be increased, as long as the biogas yield obtained in batch tests is not decreasing and a steady state between substrate addition and biogas formation is achieved. By this means the fermentation behaviour of an unknown substrate can be studied. Residence time and substrate loading rates under steady state conditions are valuable indicators for the digester scale up in technical applications.

Table 13.3. Biogas yields as measured in batch fermentation tests with diluted substrates. Required minimum residence times estimated in continuous fermentation tests. All tests performed in laboratory scale fermentation test equipment at a temperature of 35°C. $VS_{add.}$ - Biogas yield referred to the amount of volatile solids added.

Biogenic waste	Biogas yield in batch tests [$m^3 . kg^{-1} VS_{add.}$]	Minimum residence time in cont. fermentation [days]
Animal fat	1.00	33
Flotation sludge	0.69	12
Stomach- and gut contents	0.68	62
Blood	0.65	34
Food leftovers	0.47-1.1	33
Food leftovers (Fast Food)	0.693	35
Rumen contents	0.35	62
Pig manure	0.3-0.5	20
Cattle manure	0.15-0.35	20
Chicken manure	0.35-0.6	30
Primary industrial sewage sludge	0.30	20
Secondary sludge (municipal)	0.2-0.35	20
Egg residues (Pharmaceutical)	0.97	45
Blood plasma (Pharmaceutical)	1.36	45
Fermentation slops (Microbial)	0.85	35
Molasses distillery slops	0.42	14
Maize distillery slops	0.4	21
Potato distillery slops	0.47	10
Market waste	0.90	30
Municipal biowaste (Source sep.)	0.40	27
Bio-waste (31%) + Sewage sludge	0.54	30
Grey waste	0,08 - 0,15	20
Waste edible oil	1.104	30
Potato waste (Chips residues)	0.692	45
Potato waste (peelings)	0.898	40
Potato starch processing	0.35-0.45	25
Brewery waste	0.3-0.4	14
Vegetable and fruit processing	0.3-0.6	14
Chipboard manufacturing wastewater	0.893	14

3.12 Composition of biogas

Provided the elemental composition of a substrate is known, the composition of the biogas produced can be calculated (Roediger 1967; Böhnke et al. 1993), by means of the equation 13.5.

$$C_cH_hO_oN_nS_s + y.H_2O = x.CH_4 + (c\text{-}x).CO_2 + n.NH_3 + s.H_2S \qquad (13.5)$$

where $x = 1/8.(4c + h - 2o - 3n - 2s)$ and $y = ¼.(4c - h - 2o + 3n + 2s)$

Simple analytical measurement of the biogas composition can be made through CO_2 –absorption in alkaline solution. By passing a measured volume of biogas through an absorption column, the remaining gas volume roughly represents the proportion of methane (Braun 1982). Precise measurements of methane and other biogas components are performed through gas chromatography and infrared spectroscopy. Euipment for continuous on-line measurement of the gas quality is commercially available from different manufacturers.

Raw biogas (Table 13.4) typically consists of methane (50-75%) and carbon dioxide (25-50%). Depending on substrate source and processing, trace components like H_2S, NH_3, volatile hydrocarbons or siloxanes can appear in raw biogas.

Table 13.4. Chemical composition of biogas, concentrations and properties of the components.

Component	Concentration in raw biogas	Properties
CH_4	50-75% (v/v)	Energy carrier
CO_2	25-50% (v/v)	Corrosive, especially in presence of water
H_2S	0-5,000 ppm (v/v)	Corrosive, SO_2 - emissions during combustion
NH_3	0-500 ppm (v/v)	NO_x - emissions during combustion
Siloxanes	0-50 mg/m^3	Engine damage (sands off) in CHP
N_2	0-5 % (v/v)	Decreases heating value
Water vapor	1-5 % (v/v)	Corrosive

Furthermore water vapor is regularly present and nitrogen can be detected in case of simultaneous biological H_2S removal through controlled aeration.

For the energetic use of biogas in engines (combined heat and power plants) or burners, the net calorific value of the gas mixture is essential. Depending mainly on the available methane content, it can be calculated,

using the heating value of 36.14 MJ/m^3 from natural gas. Considering the water vapor content and trace gas components, a net calorific value of 21.48 MJ / m^3 results for a biogas with 60% (v/v) methane content.

For the technical use of biogas further important properties are the Wobbe index, rate of flame propagation, explosive gas mixture limits and air requirement for combustion (Table 13.5). The Wobbe index is defined as the amount of energy introduced to the burner. It is commonly used as a comparative specific value allowing the interchangeability of different gases.

For the safety of biogas installations, the combustion behaviour of biogas (methane) is most important. Within the limits of about 4.4-16.5% (v/v) CH_4 in air, the mixture burns explosively if a proper ignition source occurs. Static electricity, sparks or open fire sources can cause the ignition of the explosive gas mixture. Comprehensive safety measures have to be followed and specific national country regulations control installations and handling of biogas storage, -transport and -use in engines, burners and biogas upgrading. Construction and installation of biogas plants have to follow strict building regulations, fire prevention rules and lightning protection.

Odor prevention from biogas production, -handling and -use became increasingly important with growing distribution of biomethanation. Although emissions can arise at various stages of a biogas plant, biogas itself can develop considerable odors. Up to 500,000 odor units / m_3 have been reported to be frequently found in olfactometry measurements (Liebich 2004). Uncontrolled emissions of raw biogas, as well as off gas emissions from biogas combustion, can cause heavy odor emissions and measures have to be implemented for the prevention of uncontrolled emissions.

Table 13.5. Physical properties of biogas with an average composition of 60% (v/v) CH_4, 38% (v/v) CO_2 and 2% (v/v) trace gas components.

Parameter	Unit
Net calorific value	21.48 MJ / m^3
Wobbe index	19.5 MJ / m^3
Density	1.21 kg / m^3
Ignition temperature	700^{0}C
Explosive mixture limits of CH_4 with air	4.4-16.5 % (v / v)
Rate of flame propagation	0.25 m / second
Air requirement for combustion	5.71 m^3 / m^3
Odor[1)]	500,000 Odor units / m^3

[1)] Odor depends on concentration of specific trace gas components e.g. H_2S, volatile organic compounds

3.13 Biogas purification

From practical experience, minimum requirements for the energetic use of biogas haven been defined (FNR, 2005). The net calorific value should be above 4 kWh/m^3 biogas and the methane number should be higher than 135 (Table. 13.6). The methane number determines the pre-ignition resistance (knock rating) of a burnable gas. The value is determined by the methane content and by the overall biogas composition and properties. The methane number can be estimated in a testing engine or calculated based on a defined composition of the biogas. Increasing numbers imply decreasing ignitibility, decreasing numbers imply increasing risk of pre-ignition.

Trace gas contaminations like H_2S, chlorine, fluorine or siloxanes corrode engines in CHP installations and cause unacceptable waste gas emissions. Dust can cause deposits in the piping and clogging of burner nozzles. Excessive water vapour content in the biogas amplifies the corrosive action of CO_2 and H_2S.

Table 13.6. Minimum requirements for the energetic use of biogas in engines or burners (FNR 2005).

Parameter	Unit
Net calorific value	≥ 4 kWh / m^3 biogas
Methane number	> 135
H_2S -content	≤ 0.15% (v/v)
Σ chlorine + fluorine	≤ 100 mg / m^3 CH_4
Silicon	≤ 10 mg / m^3 CH_4
Dust (3 – 10 μm)	≤ 10 mg / m^3 CH_4
Relative humidity	≤ 90%

Depending on its projected use, a proper biogas purification usually must include dehumidifying, dedusting and H_2S removal. If gas compression or –liquefaction is envisaged, CO_2 must be removed additionally.

The removal of water vapour can be achieved through water traps, cooling or absorption. Most frequently water traps are implemented into the gas piping, providing a required humidity of less than 90 %. Dust can be safely removed by adding dust filters into the gas piping system.

For the removal of H_2S in most cases biological desulfurisation is applied. By controlled addition of air into the gas space of the digester, aerobic sulphur oxidising bacteria are proliferated and to a large extent ensure the oxidation of H_2S to elemental sulphur, e.g. *Thiobacillus thioparus*,

(equation 13.6) and sulphate, e.g. *Acidothiobacillus thiooxydans* (formerly *Thiobacillus thiooxydans*), (equation 13.7).

$$S^{2-} + ½\ O_2 + 2\ H^+ = S^0 + H_2O \quad (13.6)$$

$$2\ S + 3\ O_2 + 2\ H_2O = 2\ H_2SO_4 \quad (13.7)$$

For the safe operation of biological desulfurisation, a stoichiometric addition of air into the digester is required. Excessive aeration increases the nitrogen content, respectively decreases the heating value of the resulting biogas. Furthermore explosive gas mixtures must be safely prevented by strict control of the air addition. Depending on the sulphide content being present, the required oxygen addition can be calculated from equations 13.6 and 13.7. For medium H_2S levels commonly found in biogas, an addition of roughly 3-5% (v / v) air referred to biogas proved practically sufficient (FNR 2005).

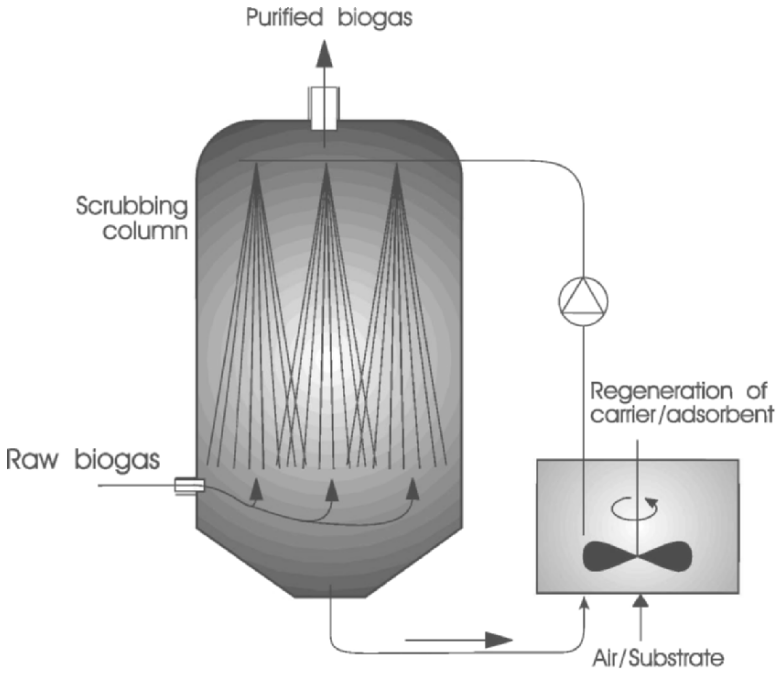


Fig. 13.7. Principal scheme of biogas purification with external bio-scrubber and regeneration unit for the circulation liquid.

To prevent uncontrolled influences on methanogenesis and for better process control possibilities, the desulfurisation process is often placed in external purification columns outside the digester. The raw biogas flows through the carrier bed containing either microorganisms, absorption fluid or solid adsorbents. While passing the column H_2S is oxidised, absorbed to a washing fluid or bound to adsorptive carriers, e.g. acitivated carbon, zeolite. After depletion, a regeneration of the washing fluid or absorbent is required (Figure 13.7).

Conventional desulfurisation processes, e.g. sewage sludge digesters, since decades use iron chloride salts for the extensive precipitation of H_2S as FeS (Seebaum 1964). Iron chloride can be directly added into the digester in stoichiometric amounts, or the biogas is passed through external columns filled with carrier materials containing precipitating iron (Dichtl and Sixt 1996).

While removing H_2S efficiently, most biogas purification processes described above, cannot remove siloxanes or chlorine- and fluorine compounds from biogas. Siloxane contaminations usually originate from solvents, lacquers, cosmetics, cleaning agents, detergents or silicone containing products. A proper selection of the substrates applied, respectively prevention of specific contaminations can be important when industrial wastes and co-substrates are occasionally applied in digestion.

For the removal of siloxanes adsorption on activated carbon or silicagel can be applied. In liquid absorption, sulfuric acid and hot concentrated nitric acid are efficient segregation media. Frequently used desulfurisation columns, based on iron beds, transform volatile siloxanes to siloxanoles and polysiloxanes. Depending on the catalytic qualities of the materials applied, a reasonable removal of volatile siloxanes can be obtained.

Numerous biogas purification plants based on the different principal systems described are commercially available. Provided proper operation, control and maintenance is guaranteed, sufficient H_2S removal for the save operation of biogas engines and –burners can be achieved. The reliable removal of impurities in biogas furthermore is the primary prerequisite for achieving the existing strict waste gas standards (Anonym 2005b) with CHP engines or burners.

3.14 Heat and power generation from biogas

Since the main component methane contributes to the global greenhouse effect, complete coverage and proper use of biogas is required. Losses of biogas in all stages of production and use must be strictly prevented. All biogas installations must include flares, being automatically ignited in case of failure of the biogas consuming unit.

Thermal use through combustion in gas burners represents the easiest application of biogas. By combustion, the energy content of biogas can be used with comparably high efficiency for heating purposes.

The use of biogas in engines of combined heat and power plants (CHP), simultaneously transfers the chemical energy of methane into electrical power (about ⅓) and heat (about ⅔). While electricity usually can be advantageously used completely, the degree of heat use in many cases is comparably small. CHPs therefore often result in very low overall energy efficiencies. Nevertheless, use of biogas through CHPs represents the most widely distributed use and numerous commercial providers offer standardised equipment. Typical installations range from 100 kW up to 3-5 MW electrical power. The achieved electrical efficiency is reported to be between 34-40 % (FNR 2005).

To prevent unacceptable exhaust gas emissions, engines in CHPs must be thoroughly adjusted to the respective fuel gas conditions. Strict limiting values for dust, CO, NO_x, SO_2, H_2S and NMHC have to be met. Among others, a German guideline (TA – Luft 2002) sets standard values for dust (<20 $mg.m^{-3}$), CO (<650 $mg.m^{-3}$), formaldehyde (<60 $mg.m^{-3}$), NO_x (< 500 $mg.m^{-3}$) and SO_2 (<350 $mg.m^{-3}$). Furthermore standard values for unburnt (NMHC) - non methane hydrocarbons - (<150 $mg.m^{-3}$) and H_2S (<5 $mg.m^{-3}$) in engine exhaust gas have been remitted (BMWA 2003).

3.15 Biogas upgrading

In order to achieve a more efficient, complete use of biogas, upgrading to natural gas quality and feeding into the natural gas grid or further upgrading to fuel quality has been realised occasionally. For achieving natural gas quality, among others, the removal of CO_2 from biogas is prerequisite, since the Wobbe index from natural gas is 54.8 MJ/m^3 compared to 27.3 MJ/m^3 in biogas (65% CH_4). Only burning gases with a similar Wobbe index can substitute each other. By removing CO_2 a acceptable Wobbe index can be achieved in biogas.

Several methods are commercially available for CO_2 removal. Most widely distributed is the pressurised water absorption of CO_2. Since methane is fairly insoluble in water while CO_2 dissolves to a high degree, CH_4 can be released in a pressurised absorption column. Dissolved CO_2, together with a major part of H_2S, is released in a second desorption column. Normally 10-12 bar at ambient temperature (20°C) are applied in the absorption process. More advanced processes replace water by chemicals (e.g. Polyglycolether), achieving higher absorption efficiencies for CO_2 and H_2S, while being more energy efficient and water saving. A methane content of 96% can be realised through pressurised water absorption.

Further processes for CO_2 removal from biogas are pressure swing adsorption, the liquefaction of biogas or the use of membranes. With pressure swing adsorption similar methane concentrations (96 %) like in absorption processes can be realised. Biogas is passed through a series of dry adsorption columns using activated carbon or molecular sieve. During alternating compression (5-10 bar) and decompression CO_2 is adsorbed respectively released, ending up with an CH_4 enriched gas after passing the column series. Several upgrading plants, based on pressurised water absorption and pressure swing adsorption, have been realised in Sweden (Jönsson 2005) and Switzerland (Wellinger 2005).

The use of membranes for CO_2 removal, as well as the liquefaction of methane so far have comparably little practical experience and significance. Polymeric membranes principally are available with specific permeability for the various biogas components (Melin and Rautenbach, 2004). Efficient CO_2 separation can be achieved and additionally water, ammonia and H_2S may be considerably reduced simultaneously. Gas permeation through membranes therefore represents a good alternative to conventional processes for gas treatment, provided reliable and cheap membrane materials will be available in the future. Nevertheless although numerous membrane separation processes are commercially offered, little practical applications have been realised for biogas separation and many developments are still in a pilot stage.

Liquefaction transfers biogas to a liquid fuel which can be easily transported, stored and most advantageously, used as fuel in vehicles. Unfortunately biogas cannot be converted to a liquid under ambient temperatures or pressure and therefore requires considerable energy input for liquefaction. The process requires in a first step biogas compression to 200 bar. After compression trace gases (e.g. H_2S) are adsorbed by passage through a molecular sieve. Thereafter the liquefied gas mixture must be separated into CO_2 and CH_4 in a cryogenic rectification process. The liquefied methane, at ambient pressure, needs to be stored at $-161^{o}C$, the pressurised CO_2 (20 bar) can be used as a by-product.

3.16 Digestate

Methanogenesis is performed by a complex mixture of symbiotic microorganisms, transforming soluble organic materials under oxygen-free conditions into biogas, nutrients and 5-10% new cell matter. Salts, refractory organic- and inorganic matter are left as by-products and remain, together with the freshly grown cell mass, as fermentation residue, i.e. digestate. Under optimum conditions, through the anaerobic treatment, as much as 90% of the biodegradable organic fraction of a substrate can be stabilised,

respectively transformed. Biomethanation of energy crops for example, transforms about 80% of the input TS into biogas, leaving just about 20% of the TS in the remaining digestate. Depending on the substrate biodegradability, the digestion conditions and on the content of refractory contaminants, the remaining residual fractions can be considerably bigger in anaerobic waste treatment. With some bio-waste for example, roughly 50% of the input waste TS can remain in the digestate and just 50% are gained as biogas. Proper handling-, treatment- and use of digestate can therefore get very complex and expensive in anaerobic waste treatment.

As a prime goal, the digestate should always be recycled to soils as a fertiliser or soil conditioner, preferably in the immediate neighbourhood of the digestion plant. For land application, the digestate quality must comply with the stringent European and National soil protection regulations. If the digestate cannot be directly applied in agriculture in the vicinity of the digestion plant, longer transport distances frequently demand for further treatment, e.g. dewatering, composting, NH_3 removal, P-removal. Big installations of waste treatment- or crop digestion plants may even be forced to liquid digestate treatment- or purification, e.g. filtration, membrane separation, reverse osmosis, aeration, and recycling of the purified water to the digestion process (Klink 2005). Through biological treatment, effluent BOD values of 20 – 400 $mg.l^{-1}$ can be achieved (Schleiss and Engeli 1997; Kautz and Nelles 1995), according to degradation efficiencies of 60-90%.

Poor quality substrates should not enter digestion processes provided for digestate recycling. Residues not meeting the required land application standards, must be further treated and disposed off otherwise, i.e. combustion, mechanical biological treatment with subsequent landfill. Further treatment requirements and longer transport distances for digestate can get costly and soon become economically prohibitive. Overall costs must therefore be carefully considered during process evaluation.

Improper handling of digestate and spreading on agricultural land can be accompanied with noise- and odor development. It may also be connected with hygienic risks and contamination of land and groundwater. For this reason, a proper control of the digestate quality and compliance with good agricultural practice for digestate application is indispensable. Only sufficiently stabilised digestate can guarantee the prevention of emissions and realise the full advantage of nutrient recycle to agriculture.

3.17 Hygienisation

Due to the potential presence of pathogenic bacteria, viruses and parasites, numerous wastes represent hygienic risks, e.g. sewage sludge, livestock wastes, slaughterhouse wastes, food leftovers. The US Environmental

Protection Agency (EPA) and the European Union have both released regulations, requiring specific treatment and handling conditions for hygienically risky wastes, prior to land application. In particular the European regulation (EC) No 1774/2002, (ABP, Animal By-Products Regulation), laying down health rules concerning animal by-products not intended for human consumption requires strict handling and treatment procedures for defined animal derived wastes. Based on their potential risk to the public, to animals, or to the environment, the ABP regulation classifies all animal by-products and their processed products and wastes into three categories and defines respective treatment and utilisation possibilities. Depending on the risk potential, waste treatment requirements can include pasteurisation (70°C, 1 hour), or even sterilisation at 133°C (3 bar, 20 min) or incineration. Animal manure, stomach- and intestine contents, milk, colostrums, or mixtures of them with other biogenic wastes or raw materials (energy crops, silage), not covered by the ABP regulation, may be processed in biogas plants without pre-treatment. The fermentation end product may be used and applied on farm or pasture land without having to meet any requirements from this regulation. Many other wastes, e.g. slaughterhouse, animal based food industry-, kitchen- and restaurant waste, have to undergo thermal treatment when used in a digestion.

The fermentation conditions (i.e. chemical composition, redox potential) of methane fermentations, in particular together with elevated fermentation temperature (55°C), show inhibitory effects on pathogenic microorganisms. Comprehensive investigations on the effects of digestion conditions have been performed in Germany (Philipp and Böhm 1997; Martens et al. 2000; Strauch and Philipp 2000; Silvers 1991), England (McKain and Hobson 1987; Carrington 2001; Carrington et al. 1991), Denmark (Bendixen and Amendrup 1992; Bendixen 1999) and elsewhere (Pesaro et al. 1995; Kearney et al. 1993; Mateu et al. 1992; Spillmann et al. 1987). It could be shown, that during digestion a sufficient hygienisation effect can be achieved, provided a minimum hydraulic residence time of 7-20 days and a minimum guaranteed retention time of 4-24 hours was kept at a digestion temperature of 52-55°C. Mesophilic digestion proved insufficient for achieving hygienisation.

The hygienisation effect of thermophilic fermentations can advantageously be used when hygienically risky wastes are to be treated. The ABP regulation also allows for the development of alternative hygienisation procedures, provided an equivalent effect to standard treatment processes can be achieved. National regulations can implement thermophilic digestion as an alternative to thermal pasteurisation.

4. Applications of biomethanation

4.1. The advantage of closed circuit processes

Biomass based technologies can be operated sustainable, as closed circuit processes (Figure 13.8). Biomass can be directly used in a multitude of applications, or it can be processed mechanically and by chemical - biological techniques to a great variety of products (Klass 1998). Since most biomass based upgrading- and production processes also release by-products and wastes, biomethanation can be advantageously implemented into these technologies as an energy- and fertiliser recovery process.

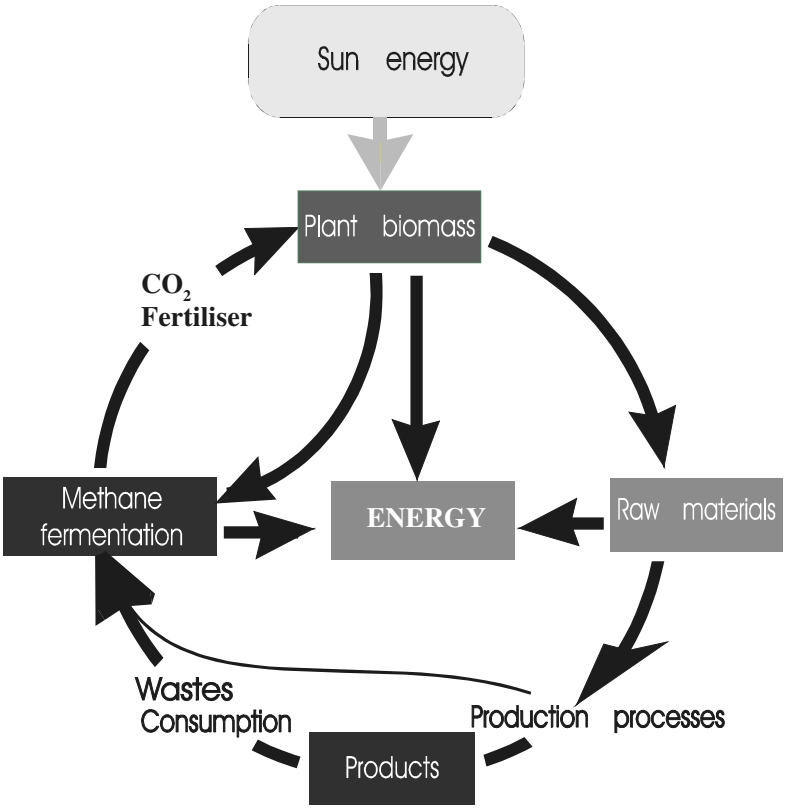


Fig. 13.8. Plant biomass and its universal role as raw material and energy source. Methanogenesis can be optimally implemented for energy recovery from biomass as well as biomass processing wastes, ultimately releasing CO_2 and nutrients for assimilation.

Biomethanation is capable to process and stabilise a big variety of wastes and by-products. On the other hand, digestion of different renewable biomass sources represents a new challenge in alternative energy supply. This feature offers an important long term potential for biomethanation in renewable energy recovery from biomass. Through the ultimate energetic use of biogas in engines or boilers, CO_2 is released to the atmosphere, which again is converted to biomass by assimilation processes in green plants. Provided only biomass based raw materials and processes are applied, no additional CO_2 will be released from this closed circuit.

Various concepts for biomass based "Biorefineries" have been proposed in the last years. Such production processes for materials, chemicals, pharmaceutical products and power generation could contribute to a sound industrial development and could offer new possibilities of rural occupation and income for farmers. With progressing depletion of fossil resources and consequently increasing energy costs, biomass based production processes and power generation will gradually become competitive to conventional fossil fuel based technologies. Due to its multi-faceted capabilities, biomethanation processes will play a universal role in future biomass utilisation concepts.

Biomethanation systems provide significant benefits to aid in meeting the increasing environmental regulations and public pressures on organic waste disposal and energy recovery. Wastewaters can be treated to yield acceptable effluents for receiving sewers and water courses. Sludges and solid wastes can be treated, resulting in stabilised end products with useful fertiliser properties. Lastly renewable raw materials i.e. "energy crops" can be processed with favourable efficiencies, yielding the directly applicable energy carrier biogas.

Many bio-process technologies or -operations cannot efficiently use the raw materials applied and hence, usually huge amounts of by-products or wastes are generated during processing. Biomethanation can advantageously be implemented into numerous common biotechnological production processes. The implementation of methanogenesis can substantially improve the degree of utilisation of the valuable raw materials through biogas- and fertiliser production from by-products and wastes. Many of these possible examples of such cascade types of raw material use can be found, for example in the food- and beverage industry, biochemical- and pharmaceutical industry or in bio-ethanol production.

4.2. Waste stabilisation

4.2.1 Sewage sludge

Anaerobic sewage sludge stabilisation today is state of the art. Waste stabilisation originally was the main goal for the historic developments of technical applications in waste water treatment. The anaerobic stabilisation of sewage sludge through digestion presumably is among the oldest technical

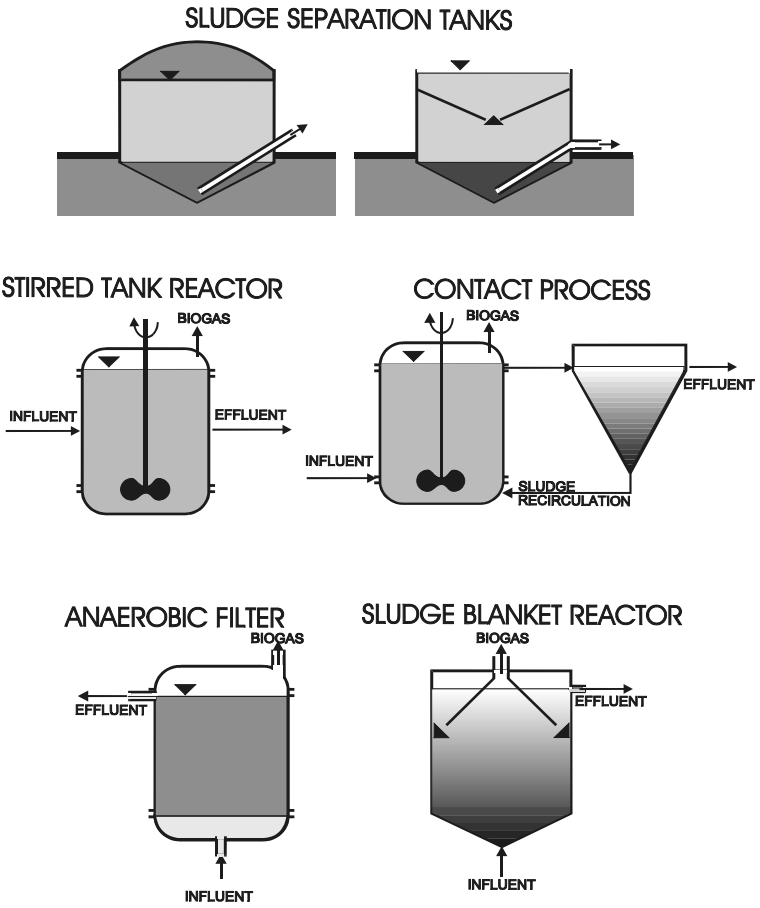


Fig. 13.9. Development of reactor design in anaerobic waste water treatment, from early sludge separation tanks (Talbot tank, Imhoff tank), stirred tank reactors to sludge enrichment through aggregation/sedimentation (Contact process, sludge blanket reactor) and immobilisation (Anaerobic filter).

applications of methanogenesis. Anaerobic treatment was first applied for municipal waste water, respectively sewage sludge from waste treatment plants. First technical applications occurred 1881-1895 in USA, UK, France and Germany (McCarty 1982). Typically the waste water was collected in tanks i.e. septic tank, Talbot tank, Imhoff tank and stored anaerobically for several weeks (Figure 13.9). The digested sludge was removed batch-wise and spread on land. Nearly all of these early applications have now gradually disappeared.

Early descriptions of more sophisticated sewage sludge digestion processes were published by Hoppe (1956) and Roediger (1967). The current state of the art of sewage sludge digestion was comprehensively described by Eckenfelder and O`Connor (1961); Cheremisinoff and Morresi (1976); Kemmer (1979); Koot (1980); Grady and Lim (1980); Dauber (1993) and the German ATV -"Abwassertechnische Vereinigung" (Leschber and Loll 1996). Typically large digester units, up to 10,000-15,000 m^3 volume, are constructed in centralised sewage treatment plants. Primary sludge, settled from the receiving waste water, is collected together with surplus sludge from the aeration basins and usually kept for about 20 days at 37°C or alternatively 10 days at 55°C digestion temperature. Depending on the degree of sludge stabilisation during aeration, the biogas yield from sewage sludge amounts 0.27-0.48 m^3/kg VS added (Leschber and Loll 1996). Annually between 6-12 m^3 biogas are obtained per inhabitant. The biogas obtained from sewage sludge digestion normally is used in CHP plants. The electricity produced thereby, in most cases can cover the overall energy demand of a sewage treatment plant.

Provided the quality standards for soil application are met, the residual sludge (digestate) can be applied as fertiliser and soil conditioner in agriculture. Frequently further sludge treatment, i.e. dewatering, drying, composting, has to be applied prior to further applications. If a direct use of the residual sludge cannot be realised, mechanical biological treatment with subsequent disposal to landfills or combustion of the digestate has to be applied.

4.2.2 Industrial waste water

Many other waste digestion applications have been directly derived from sewage sludge digestion technology. Reactor configuration based on stirred tank reactors (see Figure 13.9) were early extended with sludge recirculation from sedimentation vessels (Contact process) and applied for various industrial food- and fermentation waste waters (Schroepfer et al. 1955; Donnely 1978; Anderson et al. 1981). Through biomass retention, respectively the possible increase of the solids residence time up to

about 100 days, an increased VS degradation rate at decreasing residence times could be achieved in contact processes.

For waste waters with low suspended solids content (e.g. condensed exhaust vapours, distillery slops), fixed film systems (i.e. anaerobic filter) were developed (Young and McCarty 1969; Young 1991). Derived from aerobic trickling filter applications (Coulter at al. 1957), early anaerobic filters (see Figure 13.9) used porous lava stone fillings as a carrier for the biofilm growth. More sophisticated developments applied a variety of different synthetic materials, formed to balls, tubes or corrugated sheets of different sizes. These systems achieved considerably high biomass retention and hence, comparative low hydraulic residence times (Table 13.7). As a main disadvantage, anaerobic filter reactors are sensitive to clogging and therefore restricted to wastes low in suspended solids. Among others, applications have for example been reported from starch industry wastes (Taylor and Burn 1973; Landine et al. 1980), dairy industry (Witt et al. 1979), vegetable processing (Brondeau and Girardon 1982) and apple processing (Liqudara and Ott 1983).

Based on the phenomenon of microbial aggregate formation, the sludge blanket reactor (see Figure 13.9) has achieved a comparable wide application for industrial waste water treatment. Starting with early developments in South Africa (Cillie et al. 1969), the Upflow Anaerobic Sludge Blanket (UASB) process was further developed in the Netherlands (Lettinga et al. 1976, 1980) and subsequently investigated in using a big variety of different industrial wastes (Heertjes and van der Meer 1978; Pipyn et al. 1979; van den Berg et al. 1981; Verstraete et al. 1996). Through application of high selection pressures the formation of fast settling bacterial granules could be stimulated, allowing sludge concentrations of 150 $g.l^{-1}$ TS (see Table 13.7), low hydraulic residence times and volumetric loadings up to 25 $g.l^{-1}.d^{-1}$ COD. Reliable operation is achieved preferably with low strength wastes (COD < 10,000 $mg.l^{-1}$), low in suspended solids, Ca^{2+} ions, NH_4^+ and fat (Hulshoff Pol et al. 1983). Since the system allows the construction of comparably small and compact installations, many industries have installed sludge blanket reactor systems for waste water treatment. Numerous applications are known for sugar factory wastes (Pette et al. 1980), starch manufacture (Zeevalink and Maaskant 1984), potato processing (Christensen et al. 1984), beverage industry, fermentation- and food industry (Lettinga and Hulshoff Pol 1988; Borghans 1987; Ruppel et al. 1982; Black et al. 1974).

The principle of sludge retention, respectively immobilisation is also used for other reactor configurations in industrial waste water treatment. By using fine carriers (e.g. sand, glass), the bacterial sludge can be immobilised and retained in special reactor systems. The immobilised carriers can be fluidised through liquid recirculation, achieving thorough mixing,

mass transfer and substrate flow rates. Similar to sludge bed systems, fluidised bed reactors are susceptible to disturbance by particulate matter and need careful selection of appropriate wastes.

Through the coupling of cross flow microfiltration or ultrafiltration membranes to digesters, complete retention of particles can be achieved (Becker 1984). Membrane coupled reactors (Membrane bio-reactors) allow the enrichment of enormous sludge concentrations (See table 13.7), enabling compact reactors, with small digester volume, at extremely high volumetric loading rates. Since in fact all insoluble particles are retained by the membrane, the porous structure of the membrane is fairly susceptible to clogging and requires a steady pre-treatment of wastes, for the removal of particulate substance.

Both systems, fluidised bed- and membrane coupled reactors, have been realised in some special technical applications (Heijnen et al. 1985; Oliva et al. 1990; Becker 1983).

Table 13.7. Comparison of achievable TS contents, loading rates and residence times in different conventional and high rate reactor systems.

Reactor system	TS in reactor $g.l^{-1}$	Volumetric loading $g.l^{-1}.d^{-1}$ COD	Hydraulic residence time (days)
Stirred tank	3-5	2-5	15-25
Contact process	5-15	4-8	10-20
Sludge blanket	150	15-25	<1-5
Anaerobic filter	25-50	10-20	<1-5
Fluidised bed	90	15-25	<1-5
Membrane reactor	>150	>25	<1

Thousands of applications of anaerobic digesters have been built worldwide and various standard processes have been commercially developed. A comprehensive overview on systems, applications and markets of anaerobic industrial waste water treatment was given by Böhnke et al. (1993).

4.2.3 Solid waste

First applications in digestion of solid and semisolid wastes were realised decades ago with agricultural by-products like manure, straw and plant material residues, but did not receive broader distribution and acceptance. With increasing intensified crop production and animal breeding, biomethanation of agricultural wastes later on received renewed interest, primarily as an environmental protection technology. While odors could be considerably reduced, digestion additionally released useful biogas and

fertiliser as by-products. Reactor technology and respective applications of biomethanation in agriculture have been comprehensively described in literature (Liebmann 1956; Stafford et al. 1980; Braun 1982; Baader 1983; Wellinger et al. 1984; Kloss 1986; Kuhn 1995). Nevertheless, with the exception of a tremendous amount of small rural digester applications in China (Chen Ruchen 1981; Nyns 1986), the number of agricultural "1st generation" biomethanation applications in the developed world, until recently remained comparably small.

Increasing environmental pressure on waste disposal and increasing energy costs brought up numerous organic wastes and by-products, requiring further treatment. The stepwise introduction of landfill bans for organic wastes caused increasing treatment- and disposal costs for organic waste materials. Through co-digestion of wastes together with sewage sludge or liquid manure, the process economy of biomethanation can be considerably improved. Most organic wastes can be advantageously used for digestion. Consequently numerous waste materials are now used in digestion processes.

The vast majority of organic waste materials listed in Table 13.8, e.g. slops, spent liquors, pulp & paper wastes, oil- and fat trap wastes, can be treated in stirred tank reactor- or contact process systems, without complex pre-treatment requirements. Inhomogeneous wastes and materials bearing unwanted contaminants, e.g. source separated organic household waste, kitchen & restaurant waste, market wastes, can require comprehensive pre-treatment. Depending on the pre-treatment process applied, different appropriate reactor systems have been developed for the use in solid waste digestion. If conventional contaminant removal by sorting, sieving and crushing is applied, dry fermentation systems are used in digestion (Figure 13.10). Usually a final TS content of 20-35% results in dry fermentation systems. The wet separation process additionally separates a light fraction of contaminants (e.g. plastics, compound materials), at the surface and a heavy fraction (e.g. gravel, cullets, metals), from the bottom of an external, conical pulper. If wet separation of contaminants is applied, the resulting fermentation liquor usually contains less than 10% TS and conventional digester systems, e.g. stirred tank, contact process, can be applied for further treatment. Bioreactors used for dry fermentations, require sophisticated installations for proper substrate mixing and transport. High pressure piston pumps are applied in horizontal tank reactors, while slow moving blade stirrer equipment is installed for mixing and substrate transport in horizontal tanks.

Table 13.8. Biogenic waste materials suitable for biomethanation.

Waste material	Remarks
Biogenic materials from agriculture	
Straw and other fibrous plant residues	Chopping or grinding required
Green plant material, crops, grain, silages	Chopping required, disturbing sand, stones, scum layer formation can occur
Harvest residues	Chopping required, disturbing sand, stones
Silage leachate	High COD loading can result
Chicken manure	Inhibiting NH_3-contents can occur
Liquid piggery manure	
Cow manure	Chopping of bedding straw
Food industry waste	
Expired food	Expensive unpacking required
Dough, confectionary waste	Liquefaction (dilution) required
Whey	
Residues from canning & frozen foods	Expensive unpacking required
Residues from fruit juice production	Chopping advisable
Yeast and yeast like products	
Yeast- and cooler sludge from breweries	Increased H_2S – formation can occur
Sludge from wine production	Increased H_2S – formation can occur
Sludge from distilleries	Increased H_2S – formation can occur
Fruit-, Corn- and Potato slops	
Fermentation wastes	
Residues from animal feed production	
Expired feed	Pre-treatment case dependent
Slaughterhouse waste	
Animal fat	Scum layers can occur
Flotation sludge	Scum layers can occur
Stomach- and gut- contents	Hygienisation may be required
Blood	Obligatory delivered to rendering plants
Fish - waste	Grinding advisable
Chicken - waste	Scum layers can occur (fat, feathers)
Animal wastes	
Animal parts	Obligatory delivered to rendering plants
Animals from confiscation	Obligatory delivered to rendering plants
Carcasses	Obligatory delivered to rendering plants
Animal waste from rendering	Obligatory delivered to combustion
Wastes from plant- and animal fat prod.	
Spoilt plant oils	Scum layers can occur
Oil seed residues	Scum layers can occur

Table 13.8. (Cont.) Biogenic waste materials suitable for biomethanation.

Waste material	Remarks
Fat trap contents	Scum layers and hardening can occur
Fats	Scum layers and hardening can occur
Oil containing bleaching earth	High inert materials content
Edible fat sludge	-"-
Pharmaceutical wastes	
Proteinous wastes	Inhibiting NH_3-conc. and foaming can occur
Bacterial cells and fungal mycelium	Hygienisation may be required
Wastes from leather prod. & processing	
Leather-, tissue-, collagen fleshings	Poor degradable contents, high salt- and heavy metal (chromium) content
Pulp- and paper industry wastes	High fibre (cellulose) content, bactericidal agents from pulp additives
Municipal wastes	
Biogenic wastes	Extended impurities separation required
Garden- and yard wastes	Chopping and impurities separation
Market wastes	Chopping and impurities separation
Food leftovers from restaurants	Impurities separation (metals, plastics, bones) and hygienisation required
Catering and airport food leftovers	High inert (packaging) content; frequently obligatory incineration
Primary waste water sludge	
Surplus sludge from boil. Waste treatm.	
Oil- and fat trap wastes	Scum layers and fat hardening can occur
Sludge from gelatine production	
Sludge from starch production	
Residues from potato starch production	
Residues from maize starch production	
Residues from rice starch production	

Comparative studies show no clear overall advantages for wet- or dry digestion processes. While wet digestion requires addition of fresh water, respectively waste water treatment, dry digestion equipment (pumps, piping) frequently suffers through abrasive effects, caused by poor removal of glass and sand during pre-treatment. Depending on the input waste quality, the final digestate use after dry digestion can be restricted, due to insufficient contaminant removal. Wet digestion on the other hand can be adversely affected by scum layer- or bottom layer formation. The biogas

yield with both processes is identical and both processes meet the requested environmental standards in terms of emissions.

In a wet digestion process typically 0.135 t light fraction- and 0.044 t heavy fraction contaminants per t source separated municipal bio-waste are sorted out (Gallert et al. 2002). As final products 91 m^3 Biogas (0.11 t) and 2.7 t digestate (4% TS) result per t bio-waste. Digestate usually is dewatered and composted, delivering 0.23 t final compost per t waste. The liquid digestate fraction can be recycled at fairly high rates (75%), 25% must be discharged to the sewer (~0.5 m^3/t waste) and replaced by fresh water.

Depending on biodegradability and contaminant content of the waste organic matter, the product ratio of biogas, respectively digestate, can vary in a considerable wide range. From practical experience with a dry digestion process for example, 1 t source separated municipal bio-waste (30 % TS) yields about 20 kg (TS) contaminants, 130 kg Biogas and 850 kg digestate (15% TS). In this case just 46% of the TS was transformed to biogas and 54% of the TS remained in the digestate. Referred to the fresh waste mass (1 t) 13% was obtained as biogas, 2% as contaminants, 15% as TS (in digestate) and 70% remained as liquid digestate.

Early applications of mixed solid municipal waste digestion together with sewage sludge were reported in the USA. Digestion of urban solid waste together with sewage sludge has been already realised in a technical application in the 1950s (Ross 1954; Pfeffer 1978). Although these first installations for various reasons disappeared, comparable applications of municipal solid organic waste digestion were re-established. A comprehensive review of possible configurations and application conditions was published by Diaz et al., (1982). Numerous examples can now be found in practice (DeBaere et al. 1985; Cecchi et al. 1985; Müsken and Bidlingmaier 1994; Anonym 1997; Kern 1999; Korz 1999a; Korz 1999b; Langhans 2000; Pavan et al. 2000; Schmelz 2000; Bro 2000; Wiemer and Kern 2000; Austermann-Haun 2001; Tidden 2003; Mata-Alvarez 2003).

In the 1990s, the use of source separated municipal bio-waste for biomethanation was rapidly extending in some European countries. In 1995 about 30 different bio-waste digestion technologies had been commercially offered in Germany (Krull et al. 1995). In 1997 a review of the International Energy Agency (IEA) lists 108 bio-waste digestion plants in Europe, processing 4,241,700 tons of organic waste (Anonym 1997). In Germany 44 plants processed 1,200,000 tons of bio-waste (Kern 1999). Similar processing capacities existed in 22 centralised digestion plants in Denmark. Remarkable distributions were furthermore achieved in Sweden, Switzerland and Austria. The number of plants and their processing capacity was increasing steadily. Recent estimations count 124 large bio-waste

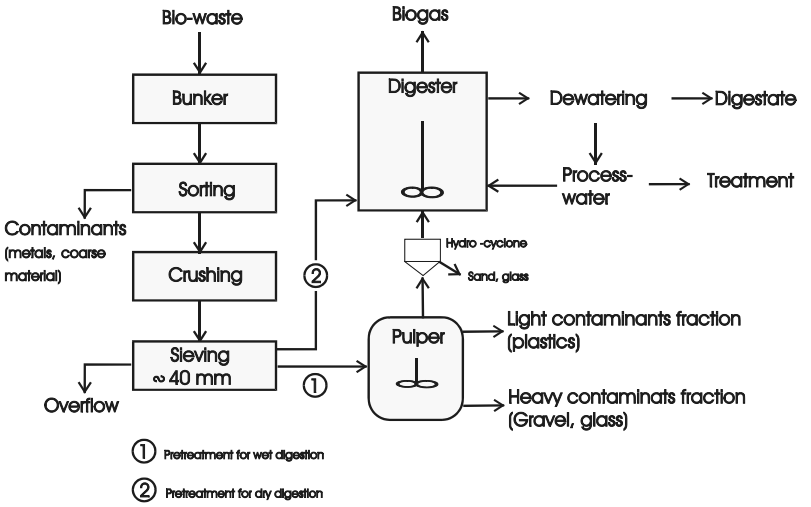


Fig. 13.10. Different pre-treatment steps for unwanted contaminant removal in wet- and dry digestion processes of solid organic waste materials.

digestion plants in Europe (DeBaere 2005), with a total processing capacity of 3,905,000 tons. The mean processing capacity of bio-waste treatment plants increased from about 20,000 t/a in 1995, to about 30,000 t/a in 2005. Compared to the overall municipal waste generation of nearly 200 million tons in Europe (Anonym 2001), the re-use rate actually still is fairly low and a huge potential for further distribution of waste recovery through biomethanation is available.

With increasing experience from municipal bio-waste digestion, further practical applications were realised, treating organic wastes from a broad variety of industries (see Table 13.8). Food industry wastes (e.g. residues from food processing), expired or spoilt food, fermentation slops and wastes (e.g. citric acid, yeast, ethanol), plant oil residues, fat trap contents, pharmaceutical wastes and even leather processing- and pulp and paper wastes are being treated anaerobically (Märkel and Stegmann 1994; Schön 1994; Kuhn 1995; Gessler and Keller 1995; DeBaere 1999; Hoppenheidt and Mücke 1999; Kirchmayr et al. 2000). A comprehensive overview on processes and applications has been published by Thomé-Kozmiensky (1995). Numerous operational experience reports from applications are available (Wiemer and Kern 1996, 1998, 2000).

For hygienic reasons, industrial wastes derived from livestock farming or processing of animal raw materials require special attention. Since inappropriate processing standards and the use of rendered products and catering

waste are believed to be the main reason for major pandemic outbreaks of BSE (Bovine Spongiform Encephalopathy) and FMD (Foot and Mouth Disease), consequently rigorous measures were taken by the European authorities. The Regulation (EC) No 1774/2002, laying down health rules concerning animal by-products not intended for human consumption (ABP, Animal By-Products Regulation), stipulates respective treatment procedures (i.e. incineration, sterilisation, hygienisation) for wastes derived from animals. Among the materials classified in 3 risk groups, just animal manure, stomach contents, milk, colostrum and dairy products can be used without any heat treatment in biogas plants. All other animal derived food- and feed waste, including kitchen- and restaurant waste, have to undergo appropriate hygienisation- or sterilisation pre-treatment. Biogas plants treating the latter animal by-products, must implement a hygienisation (or sterilisation) step and must guarantee special operating conditions (Braun and Kirchmayr 2004; Kirchmayr et al. 2004).

4.3 Energy recovery

4.3.1 Early investigations with energy crops

The idea to energetically use renewable plant biomass or "energy crops" for biomethanation is not new. Early investigations on the biomethanation potential of different energy crops and plant materials have been carried out in the 1950s by Reinhold and Noack (1956) in Germany. Stewart (1980) described the potential use of oats, grass and straw, resulting in methane yields of 170-280 $m^3.t^{-1}$TS. Investigations with seaweeds (Yang 1981; Ryther and Hanisak 1981; Fannin et al. 1982), water hyacinth (Chin and Goh 1978) and fresh water algae (DeRenzo 1977) showed medium methane yields between 150-240 $m^3.t^{-1}$TS.

Although the principal performance of crop digestion could be clearly shown, the process was rarely applied practically. Harvest efforts were expensive, the operation expense for plant pre-treatment and for proper digestion conditions were high, the conversion efficiencies of the crops used for biomethanation were fairly low and hence, crop digestion was commonly not considered to be economically feasible. With the exception of pilot investigations (Stewart 1980), dedicated technical applications of energy crop digestion were not realised in the 1980s. Crops, plants and plant waste materials were by the most occasionally added to waste based digesters.

With steadily increasing oil prices and improved economic framework conditions, "energy crop" - research and development was stimulated again in the 1990s. Numerous plants and plant materials were investigated on their methane formation potential (Table 13.9). In principal many varieties of grass, clover, corn, maize, rape or sunflower proved feasible for methane recovery. Even hemp, flax, nettle, miscanthus or potatoes, beets, kale, turnip, rhubarb and artichoke were tested successfully. A fairly wide range of methane yields from these crops, between 120-658 $m^3.t^{-1}$VS, can be found in literature. Main influential factors on the gas yield are the lignocellulose content and the phsysiological state of the plant on harvest. High fibre content slows down biomethanation (Edelmann 2001) and requires high residence times and pre-treatment (chopping) of the plant material. The influence of the harvest time and maturity of the plant material on the methane yield was investigated comprehensively (Pouech et al. 1998; Amon et al. 2003; Eder 2005). Accordingly, a decrease of the methane yield at late harvest times was observed in tests with clover grass, wheat and different maize varieties.

Table 13.9. Biogas-, respectively methane production potentials, as investigated in digestion tests with various plant- and crop materials.

Plant / Crop	Methane yield $m^3.t^{-1}$ VS	Reference
Maize (whole crop)	397	Pouech et al. (1998)
	205-375	Amon et al. (2003)
	450	Unterlerchner (2006)
Wheat (grain)	384	Pouech et al. (1998)
	426	Unterlerchner (2006)
Oats (grain)	250	Kaparaju et al. (2002)
	295	Pouech et al. (1998)
Rye (grain)	283-492	Heiermann et al. (2002)
Barley	353-658	Heiermann et al. (2002)
Triticale	337-555	Heiermann et al. (2002)
Sorghum	295-372	Pouech et al. (1998)
Straw	242-324	Reinhold and Noack (1956)
Leaves	417-453	Reinhold and Noack (1956)
Chaff	270-316	Reinhold and Noack (1956)
Grass	298-315	Zauner and Küntzel (1986)
	414	Unterlerchner (2006)
	467	Reinhold and Noack (1956)
Clover grass	290-390	Amon et al. (2003)

Table 13.9. (Cont.)

Plant / Crop	Methane yield $m^3.t^{-1}$ VS	Reference
Red clover	300 350	Lähtomäki et al. (2003) Unterlerchner (2006)
Clover	345 350	Reinhold and Noack (1956) Pouech et al. (1998)
Alfalfa	340-500	Heiermann et al. (2002)
Sudan grass	213-239 303	Amon et al. (2003) Kaiser (2004)
Red Canary Grass	340-430	Lähtomäki et al. (2003)
Hemp	409 355-377	Linke et al. (1999) Kaiser (2004)
Flax	212	Heiermann et al. (2002)
Nettle	120-420	Lähtomäki et al.(2003)
Ryegrass	390-410	Pouech et al. (1998)
Miscanthus	179-218	Kaiser (2004)
Sunflower	177 154-335 400	Heiermann and Plöchl (2004) Amon et al. (2005) Unterlerchner (2006)
Oilseed rape	240-340	Zubr (1986); Pouech et al. (1998); Heiermann et al. (2002); Weiland (2003)
Jerusalem artichoke	300-370	Lähtomäki et al. (2003)
Peas	390	Gunaseelan (2005)
Rhubarb	320-490	Lähtomäki et al. (2003)
Turnip	314	Stewart (1992)
Kale	240 310-320 334	Badger et al. (1979) Lähtomäki et al. (2003) Weiland (2003)
Potatoes	276-400	Weiland (2003)
Sugar beet	236-381	Kaiser (2004)
Fodder beet	420 456 500	Stewart (1992) Weiland (2003) Heiermann et al. (2002)

4.3.2 Development of energy crop digestion

Based on existing digesters and process technology, in the initial phase of energy crop digestion in the 1990s, many biogas plants started occasional feeding of different crop materials. Maize- and grass silage, corn and corn residues, grass cuttings were fed together with manure and different wastes e.g. grease, whey, vegetable processing waste, potato residues (Weiland 2003; 2004). The majority of the early applications to a major part used manure and only little crop material was fed together with other co-substrates. One and two step digester installations were used at hydraulic retention times between 30-150 days, according to volumetric loading rates between 1-4 $kg.m^{-3}.d^{-1}$ VS. As in many conventional digesters, the substrate was fed either once or twice a day. Crops were often fed just on occasion or when available during the harvest season. The resulting biogas was used in CHP installations and the digestate in most cases could be used next to the plant as fertiliser. Sound economic operation of the early energy crop digestion plants could not always be achieved. Considerable addition of waste materials was often necessary, to improve the process economics through the gate fees paid for waste treatment. But sufficient organic waste material was not always available, while the increasing substrate demand further reduced the gate fees paid for waste organic matter.

Changing framework conditions in agriculture and most important, continual increasing energy prices, later on were the main driving forces for a significant improve of the economics in biogas production from crops. Due to a widespread agricultural overproduction, decreasing prices of crops and increasing areas of set aside land, biomass became available for non food use at economically attractive costs. But it was not before subsidies were introduced and guaranteed feed in rates for electricity to the grid were realised, that energy crop digestion in fact became economically viable. While the number of agricultural digester applications had been constant, or even decreasing for many years, a sudden increase was recorded in the late 1990s, in Germany, Austria and to a minor extent in some other European countries. The number of biogas plants in Germany for example, increased from about 100 in 1990 to more than 3,000 installations in 2005 (Weiland 2005).

The steady increase in energy crop digester applications in Germany and Austria can be directly attributed to the favourable supportive European and National legal frameworks of eco-tariffs paid for renewable energy (Anonym 2002). Depending on the plants electrical power capacity, staggered feed in tariffs are guaranteed for the whole depreciation period of the

investment. Similar subsidising systems exist in Germany and are discussed in Switzerland. Other European countries apply tax exemptions (e.g. Sweden) for renewable energies. A supportive European framework for renewable energy has been created by the European Commission recently (Anonym 2001). Member countries are recommended to follow the goals of increasing the share of renewable energy in power supply of 22% by 2010.

4.3.3 Energy crop digestion technique

A steady technical development of energy crop digestion plants can be observed during the last years. Although numerous different commercial digester concepts have been realised in the past, a fairly comparable process technology emerged finally. As do all digestion plants, energy crop installations comprise the same main process steps, of substrate pre-treatment and storage (1), digestion (2), digestate use (3) and energy recovery (4) from biogas. A generalised process scheme is shown in Figure 13.11. Since most crops used have to undergo pre-treatment und must be stored for a year-round use, major differences to conventional digestion plants occur in substrate preparation and –storage. Due to the high TS- and fibre content in commonly applied crops or silages, various appropriate pre-treatment- and feed techniques have been developed for the use in energy crop digestion. The realisation of sufficient mass transfer in high TS containing digesters, still is a challenge for engineering development. Numerous stirrer types and mixing devices have been introduced to scope with high viscous, fibrous crop digestion requirements. Reactor design and –configuration basically rely on conventional one- and 2-step reactor solutions, using huge storage tanks for final storage of the digestate. When ever possible, the digestate is directly used as a fertilizer in agriculture. Increasing plant size and lack of sufficient fertilizing areas, more and more demand a further digestate upgrading, i.e. dewatering, drying, composting. Since an extensive use of the waste heat from power generation in CHPs is often fairly poor, better energy use efficiency through upgrading of biogas for feeding into the natural gas grid or transport fuel generation is getting increasingly popular.

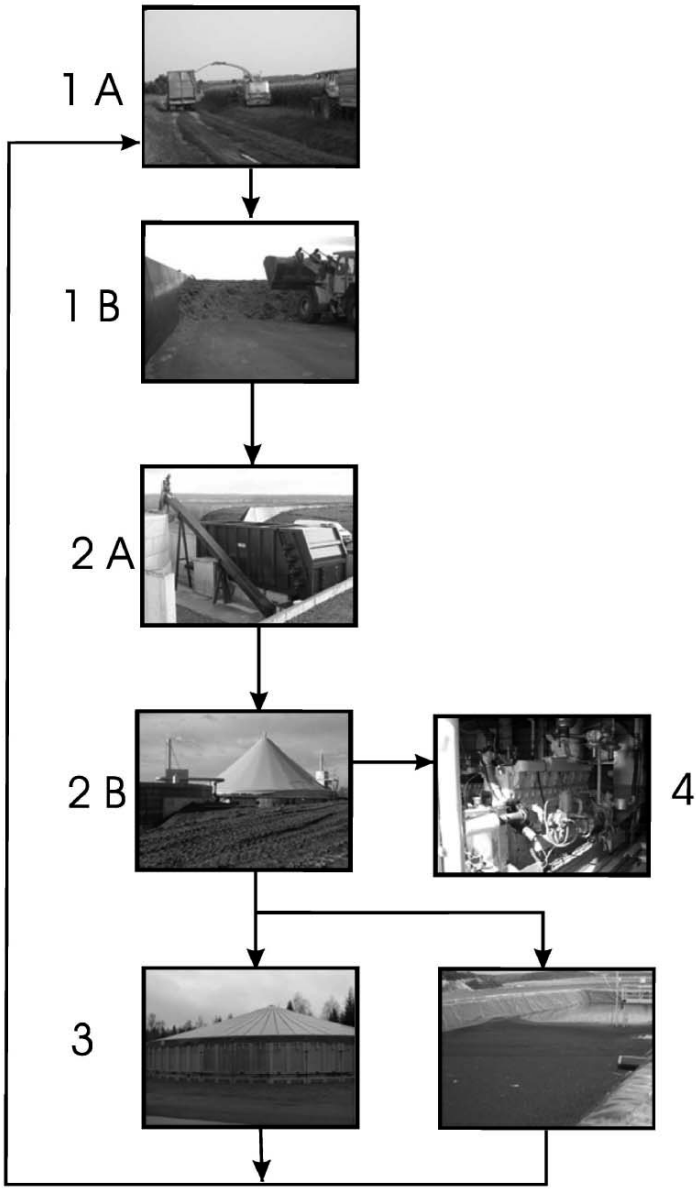


Fig. 13.11. Process steps of energy crop digestion with harvest (1 A), ensiling (1 B), substrate dosage (2 A), digestion (2 B), digestate storage (3) / –use and energy recovery (4).

4.3.4 Plant substrate pre-treatment and storage

Silos must have sufficient storage capacity for a continuous digester operation over the year. In a medium sized installation, typically up to 10,000 tons of silage are prepared during harvest time, for continuous use as substrate over the year. After harvest (1 A) the crop material is delivered to the plant, weighed and chopped before ensilage. For the storage in clamp silos (1 B), shovel loaders fed and compact the material into the silos. To prevent oxygen penetration, the material must be thoroughly covered airtight by means of plastic blankets. Alternatively high capacity bag silos (e.g. 3.5 m diameter, 100 m length), are applied. A typical bag silo storage capacity is for example about 6,000 tons of silage. Bag silos are filled with packing machines with up to 100 t / h loading-, respectively 50 ha/d harvest capacity. In smaller energy crop digestion plants, conventional big bale silos are applied for silage preparation and storage. In some cases dry storage of substrates is possible (e.g. maize corn), occasionally substrates are dried for storage, using available waste heat from the CHP.

Crop materials for ensilage should ideally have TS contents between 30-40%. Materials with TS contents below 20% result in bad silage qualities, high leachate accumulation and subsequently poor biogas yields. Regular ensiling results in rapid lactic acid fermentation and decrease of pH to 4-4.5 within several days. Typically 5-10% lactic acid and 2-4% acetic acid are formed. Butyric acid formation usually is prevented by a fast pH decrease. Acid addition, or commercially available ensiling additives, can accelerate the lactic acid fermentation and prevent silage failures. Under such conditions, silage may be stored for many months, without major damage or losses.

Further pre-treatment, e.g. hydrolysis, thermal disintegration, enzymatic degradation, have been proposed for improving ensilage and degradation rate, respectively biogas yield from crops (Lähtomäki et al. 2005; Neureiter et al. 2005). Even additives for the silage preparation (starting cultures), to affect increased storage stability of silages are commercially available (Winkelmann 2005). Broad practical experience on supporting effects of extended pre-treatment and additive application is not available so far. Since considerable mass losses up to 15% can occur during improper silage storage (McDonald 1981), adequate preparation- and application measures must be followed strictly.

4.3.5 Plant substrate feeding and digestion

Crops or silage to be fed into the digester, frequently have TS contents of at least 20% up to 40%. Dry crop material can have even higher TS contents up to 90%. Such materials cannot be pumped or homogenised with conventional digester equipment.

In conventional digestion plants, feeding plant materials usually in minor amounts, mixing tanks are applied, where the crop material is suspended with digester liquid or other liquid substrates prior to conventional dosage with piston-, displacement- or rotary pumps. Some installations use more sophisticated liquid suspension feeding, applying continuous automatic substrate dosage and control.

Large scale commercial energy crop digestion plants mainly use solid substrate feeding hoppers or container dosing units (2 A). Feed hoppers or containers are periodically filled with shovel loaders (e.g. once daily) and the material is continuously augered through gas tight auger tubes into the digester. Some applications use piston pumps instead of augers.

Most energy crop digester units consist of two – step, stirred tank reactors with integrated gas storage (2 B). Digester mixing of the high solids containing substrates in most cases is done my means of mechanical stirrers. Sufficient mixing can be achieved up to TS contents of about 10 %. Higher TS contents demand substantially increased mixing power and homogeneous digester conditions cannot always be guaranteed. Liquid digestate frequently is used for the dilution of fresh substrate. Nevertheless scum layers often cause malfunction of digesters. Phase separation usually can be prevented by an increase of substrate dilution, respectively lower TS content and loading rate.

4.3.6 Digestate storage and use

The digestate was originally stored in open tanks or lagoons (3). Depending on operational conditions, considerable residual methane formation potentials can be detected in digestate. From 11 full scale digestion plants investigated, about ¼ showed residual methane formation potentials in the stored digestate of 0-5%, ⅓ showed 5-10%, the remaining caused even higher CH_4 - emissions from 10-25% (Weiland 2005). An increasing number of plants therefore is using closed digestate storage tanks, collecting the emitted biogas for further use. Reliable prevention of methane emissions during digestate storage- and application can be achieved by a proper control and limitation of the substrate feeding rate. By this means sufficient VS degradation efficiencies of 80-90% can be realised, causing only negligible storage emissions from the digestate.

Depending on plant size, available fertilising area, fertiliser demand and climatic season, the digestate has to be stored for several months and storage tank capacities of several thousand cubic meters are required.

Increasingly, digestate has to be further processed, since adequate direct use as fertiliser is not available in many large scale energy crop digestion plants. Processing most frequently consists of dewatering, with subsequent composting or drying of the solid fraction. The liquid fraction to a high

degree is recycled to the digestion process, guaranteeing sufficient dilution of the substrate fed into the digester. Surplus water has to be further treated. Nutrient recovery (e.g. ammonia, phosphate), biological purification and membrane separation processes are applied. By means of combined decanter-, sieve-, micro- and ultrafiltration processes, the COD of the digestate was reduced from 65,217 to 4,952 $mg.l^{-1}$ (Klink 2005). Subsequent reverse osmosis reduced the COD to a final value of 27 $mg.l^{-1}$. Practical applications proved the process economically viable.

4.3.7 Practical operational experience of full scale energy crop digestion plants

Case studies

A typical medium sized energy crop digestion plant is processing about 10,000 t substrate per year. Most frequently substrate mixtures are used, e.g. 4,409 t/a energy crops (Maize, wheat, rye, alfalfa), 602 t/a agricultural residues (vegetable-, beet sugar residues) and 5,500 t/a manure (Figure 13.12). In the example shown, 10,511 t/a substrate are processed (Lindorfer 2005b), using a two step, stirred tank reactor design (3,850 m^3 total volume) at 39°C digestion temperature. About 29 t/d substrate are added, resulting in a mean residence time of about 130 days. The digestate (8,600 t/a) is collected in a storage tank with 4,300 m^3 capacity, enabling 4-5 month of storage. In the respective plant, the digestate can be applied as fertiliser on nearby agricultural land.

The biogas is collected in a 950 m^3 gas storage tank and continuously used for power generation (500 kW_{el}). From 1,840,000 m^3/a Biogas, 3,718 MWh/a electricity and 1,600 MWh/a heat can be recovered.

From the amount of substrate added annually (10,511 t with 26.5% TS content) and the remaining digestate (8,600 t with 5.9% TS content), a substrate degradation efficiency of about 82% can be calculated. When the biogas produced annually (1,840,000 m^3) is referred to the substrate added (2,790 t TS with 95% VS content), a biogas yield of 694 m^3/t VS (52% CH_4 -content) can be calculated.

Comparable efficiencies were measured in a full scale energy crop digester, using maize silage as the sole substrate source (Resch 2005). Fresh silage (20 t/d with 31% VS content) is fed to a two step (each 1,500 m^3) stirred tank digester at 49°C digestion temperature. At the high residence time of about 150 days, a VS degradation efficiency of 84,5-93% was measured. The resulting biogas yield was between 700-770 m^3/t VS (52% CH_4-content).

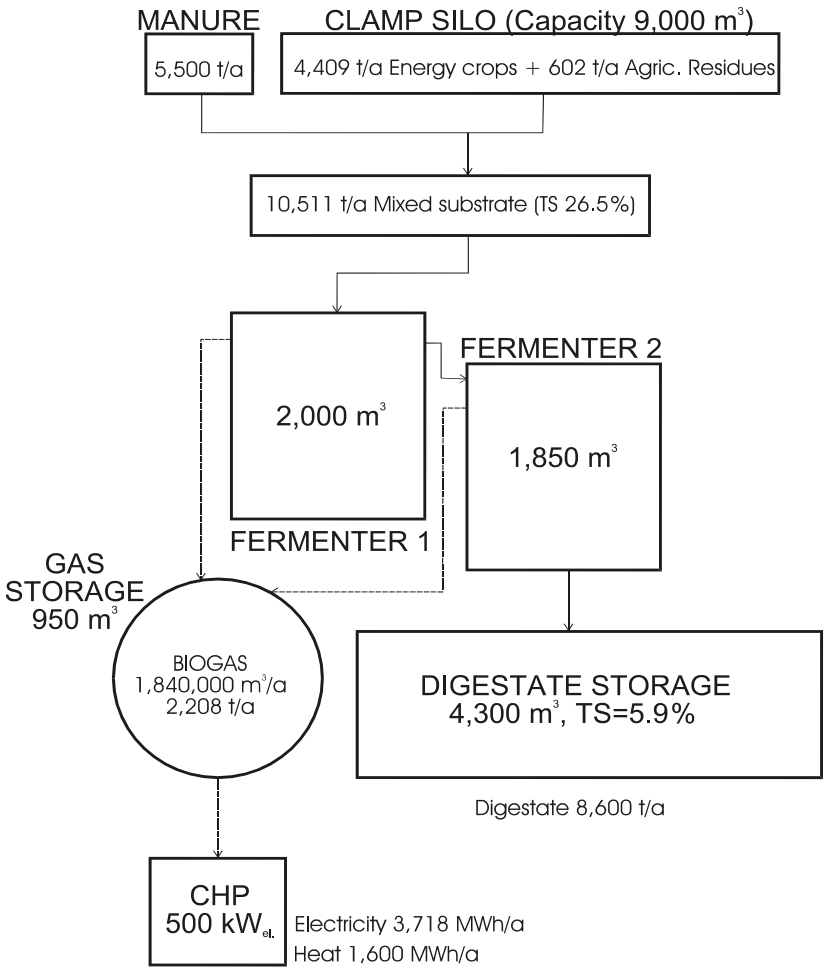


Fig. 13.12. Flow sheet and mass balance of a typical, medium sized, energy crop digestion plant (tons represent fresh mass of substrates).

Numerous less detailed case studies of energy crop digestion are reported in literature. Use of maize silage, as single substrate for example, was possible in long term full scale digestion at residence times of 80-100 days (Eder 2005). The dry matter content of the input silage (30% TS) was degraded by 50%, resulting in a pump- and mixable TS content of 15% in the reactor. The maize silage C:N:P- nutrient ratio was sufficient for a reliable, continuous single substrate digestion process.

A mixture (5,000 t / year) of maize silage, grass/clover and manure is processed to 2.4 GWh/year electricity and 3.4 GWh/year heat energy in a two – step 3,000 m^3 stirred tank reactor (Ebeling 2005). Operational parameters are 50 days residence time, a volumetric load of 3.5 $kg.m^{-3}.d^{-1}$ VS, a TS content in the reactor of 11% and a digestion temperature of 38°C. The waste heat of the CHP can be used for heating in the immediate neighbourhood. The digestate can be sold as fertilizer in the vicinity, provided the transport distance is less than 4.5 km.

A big variety of crops, i.e. triticale/rye, maize/sunflower, wheat, is processed in another example of a two step agricultural crop digestion plant (1,600 m^3). About 10-12 t / day silage (TS 25-35%) are added as the sole substrate, resulting in a final TS content in the digester of 6-7 % and a methane content of 50% (Fischer and Krieg 2005).

Waste from yeast production (17,000 t / year) is digested together with 55,000 t / year maize / winter rye, harvested from 1,300 ha (von Felde 2005). Two lines of digesters (total volumes 20,000 m^3) are installed, working at 60 days residence time and 41°C. Mixing of reactor contents (7-8% TS) is realised with pressurised biogas. In order to prevent scum layer formation, the raw materials are processed through chopping to a maximum size of 3 mm prior to digestion. From 4.2 MW CHP, 33 GWh / year electricity and 35 GWh/year heat are recovered. 50,000 t per year of digestate (8-9 % TS) is recycled as fertiliser to farmland.

Ley crop silage (5,000 t / year) is digested together with source separated municipal bio-waste (14,000 t / year) and 4,000 t / year fat trap contents, in the city of Västeras, Sweden (Nordberg 2005). The digestate can be recycled to the farms. Biogas is upgraded as a vehicle fuel.

A typical plant layout can be seen in Figure 13.13. The picture shows the two step digester system with an integrated gas storage of the second step and a separate gas storage in the background. In the foreground the covered digestate storage tank can be seen.

Biogas accumulated during final storage, contributes between 5-15% to the overall gas production of the plant.

4.3.8 Practical operational experience monitoring

In Table 13.10 the performance figures of 41 representative full scale Austrian digestion plants, i.e. capacities, operational parameters, yields, are represented (Laaber et al. 2005). In the plants considered, a fairly broad variety of substrates is applied. Energy crop addition varied between 10-100%. Five plants were not using any crop material, while 4 used crops exclusively.

Fig. 13.13. Photograph of a typical two step stirred tank reactor energy crop digestion plant, showing the covered digestate storage tank (foreground), a separate gas storage and an integrated gas storage, together with the main digester (Foto by courtesy of Ing. Pfiel, Rohkraft, Reidling, Austria).

The share of manure was 5-95 %, two plants used no manure at all. Most plants were adding agricultural residues and by-products in minor amounts (5-10 %), in 5 digesters 20-60 % were added and one plant was using agricultural residues exclusively. Bio-waste from source separated collection (mainly kitchen and restaurant waste) was digested in 11 plants (15-25 %) and 1 plant was operated exclusively with bio-waste.

Fresh substrate processing capacity varied in a wide range of about 1-59 t/d. A mean biogas yield of 0.67 $Nm^3 . kg^{-1}$ VS and a CH_4 – content of about 55 % was monitored. The VS degradation efficiency varied between 61.5-96.8 %, with a median value of 82.8%. While power generation showed median efficiencies of 31.3 %, the median thermal efficiency was fairly poor (16.5 %). As a result, the overall efficiency of the biogas energetic use was just 30.5-73%.

Table 13.10. Range of long term operational data and results from 41 full - scale energy crop digestion plants in Austria.

Parameter	Unit	Median[1]	Minimum	Maximum
Fresh substrate processing capacity	t . d^{-1}	13.2	0.8	58.9
Hydraulic retention time[2]	d	133	44	483
Loading rate (VS)	Kg . m^{-3}.d^{-1}	3.5	1	8
Amount of VS fed into digester	t . d^{-1}	2.3	0.3	13.8
Amount of biogas produced	Nm^3. d^{-1}	1,461	232	8,876
Biogas yield referred to VS	Nm^3. kg^{-1}	0.673	0.423	1.018
Biogas productivity	Nm^3 . m^{-3}.d^{-1}	0.89	0.24	2.303
Methane concentration	% (v/v)	54.8	49.7	67.0
Methane yield referred to VS	Nm^3. kg^{-1}	0.362	0.267	0.567
Degradation of VS	%	82.8	61.5	96.8
Average CHP usage rate	%	83.3	35.7	98.2
CHP operational hours per year	hours	7,300	3,100	8,600
Electrical efficiency	%	31.3	20.7	39.2
Thermal efficiency	%	16.5	0.0	42.6
Overall efficiency of biogas energy[3] use	%	47.3	30.5	72.7

[1)] Instead of average values the statistic term median is used in calculations
[2)] Mass of substrate (t /d) instead of (m^3/d) is referred to the reactor volume (m^3)
[3)] Net calorific value

A similar, earlier evaluation of German biogas plants (Weiland 2004) showed comparable results. Most plants were using manure based substrate mixtures, with different share of energy crops (i.e. maize, grass, cerials). Food- and vegetable wastes, potato processing residues, whey and fat trap contents were applied as co-substrates together with manure. Manure was dominating (75-100% share) in nearly 50% of the plants considered. An even distribution between one- and two step digester configurations was observed. In some cases (15%) three step digesters were used. Nearly 90% of all plants are operated at mesophilic temperatures. A loading rate of 1-3 kg.m^{-3}.d^{-1} VS was used in most cases. The majority of digesters were operated at residence times below 90 days (65%), the remaining used a residence time between 90 and over 150 days. The CH_4 – content in the biogas was 50-55% (55% of the plants) and 55-65% in 45% of the plants considered. The majority of all plants (80%) achieved a CHP efficiency of 80-95%, only 20% could not realise more than 50-80% efficiency.

4.3.9 Methane yields per hectare

Main criteria to select a crop for “biogas” - energy production are the yield per hectare and the expenditure (i.e. energy, labour, fertiliser) for cultivation,

harvest and processing. Furthermore the digestibility and the methane yield obtainable from the crops produced, are influencing the overall process performance and –economy. The digestibility and hence the overall yield, depend on the biomass composition. As a general rule, increasing lignocellulose content decreases the digestibility. Starch- and soluble sugar containing plants increase digestibility and degradation rates.

Table 13.11. Range of reported crop yields, range of respective methane yield measurements and range of calculated energy potentials per hectare.

Plant / Crop	Crop yield[1] $t \cdot ha^{-1}$	Measured methane yield[2] $m^3 \cdot t^{-1}$ VS	Calculated methane yield $m^3 \cdot ha^{-1}$
Maize (whole crop)	9-30	397-618	3,573-18,540
Wheat (grain)	3.6-11.75	384-426	1,382-5,005
Oats (grain)	4.1-12.4	250-365	1,025-4,526
Rye (grain)	2.1	283-492	594-1,033
Barley	3.6-4.1	353-658	1,271-2,698
Triticale	3.3-11.9	337-555	1,112-6,604
Sorghum	8-25	295-372	2,360-9,300
Grass	12-14	298-467	3,576-6,538
Red clover	5-19	300-350	1,500-6,650
Alfalfa	7.5-16.5	340-500	2,550-8,250
Sudan grass	10-20	213-303	2,130-6,060
Red Canary Grass	5-11	340-430	1,700-4,730
Hemp	8-16	355-409	2,840-6,544
Flax	5.5-12.5	212	1,166-2,650
Nettle	5.6-10	120-420	672-4,200
Ryegrass	7.4-15	390-410	2,886-6,150
Miscanthus	8-25	179-218	1,432-5,450
Sunflower	6-8	154-400	929-3,200
Oilseed rape	2.5-7.8	240-340	600-2,652
Jerusalem artichoke	9-16	300-370	2,700-5,920
Peas	3.7-4.7	390	1,443-1,833
Rhubarb	2-4	320-490	640-1,960
Turnip	5-7.5	314	1,570-2,355
Kale	6-45	240-334	1,440-15,030
Potatoes	10.7-50	276-400	2,953-20,000
Sugar beet	3-16	236-381	708-6,096
Fodder beet	8-34	401-500	3,208-17,000

[1)] Statistics Handbook Austria 2005. Statistik Austria, Vienna Austria.
[2] For data source of the range given, see table 13.9

Generalised figures for crop yields are difficult to assess. At first, growth conditions (soil type, climate, vegetation period) influence the yield. Secondly, the crop composition and yield are influenced by the physiological state of the plant at harvest. Thirdly, the overall yield figures depend on which part of the plants are usable, i.e. whole plant, roots, crops. As a matter of fact, crop yield data vary to a great extent with regional and climate conditions. Even temporary, seasonal influences (e.g. drought, frostiness), can change yields severely.

Plant breeding on the other hand is continuously improving the crop yields. Maize yields for example could be doubled during the past 50 years (Degenhart 2005). Actually the maize yields have been increased by 0.15-0.2 t/ha, per year. Mean hectare yields (dry mass) in Germany were 20.8 t in 2002, 18.7 t in 2003 and 20.9 t in 2004. Even higher yields of 25-27 t TS/ha (Landbeck 2005) and 30 t TS/ha (Eder 2005) were reported with maize varieties in Germany.

Planting experiences and growth yields from a 5 years investigation with perennial plants are reported from Atzema (2005). Yields varied between 2.1-8 t TS/ha (Red Canary Grass) and 2.2-7.7 t TS/ha (Switchgrass). Grass from nature reserves gave 1.4 t TS/ha (van Doren 2005).

The crop yields listed in Table 13.11 represent mean values, typically obtainable under central European conditions (Anonym 2005c). The biogas (methane) yields used for the calculation of the energy yields per hectare, are derived from numerous laboratory investigations (fermentation tests) on the Biological Methane Potential (BMP) of plant materials (see Table 13.11). As can be seen, the variation of the crop yields is much bigger than the respective biogas (methane) yields. For this reason, the overall energy yield per hectare primarily depends on the crop yield achievable under the respective local conditions and must be thoroughly evaluated case by case.

If using mean values, the highest crop yields are obtained with potatoes, kale, fodder beet, maize, sorghum, miscanthus and grass. The order of mean methane yields on the other hand is maize, barley, fodder beet, triticale, alfalfa, wheat and rhubarb. As the crucial final measure, the calculated methane yield per hectare is essential. It is highest for potatoes (10,258 $m^3.ha^{-1}$) and maize (9,886 $m^3.ha^{-1}$), followed by fodder beet (9,450 $m^3.ha^{-1}$), kale (7,318 $m^3.ha^{-1}$), sorghum (5,503 $m^3.ha^{-1}$), grass (4,972 $m^3.ha^{-1}$), hemp (4,584 $m^3.ha^{-1}$) and ryegrass (4,480 $m^3.ha^{-1}$). The lowest calculated median yields can be found with rye (814 $m^3.ha^{-1}$), rhubarb (1,215 $m^3.ha^{-1}$), oilseed rape (1,442 $m^3.ha^{-1}$) and peas (1,638 $m^3.ha^{-1}$).

Based on laboratory methane potential determinations, Lähtomäki et al. (2003) calculated methane yields per hectare for grass hay (2,490-2,840 m^3), red clover (1,070-1,340 m^3), marrow kale (1,730-2,300 m^3), lupine (1,150-1,720 m^3), and red canary grass (2,970-3,300 m^3).

Using 5 maize varieties in practical investigations, Amon et al. (2003), report methane yields per hectare between 3,743 – 8,529 $m^3.ha^{-1}$. Similar yields were shown by Eder (2005). Maize yielded between 4,200-8,800 $m^3.ha^{-1}$, wheat 3,000-4,000 $m^3.ha^{-1}$, barley 2,600-3,800 $m^3.ha^{-1}$ and sugar beet 5,700 $m^3.ha^{-1}$. Variations in maize yields occurred between northern Germany climate regions 4,000 $m^3.ha^{-1}$ and southern Germany 5,000-7,000 $m^3.ha^{-1}$. The influence of the maturity state on the gas yield was considered significant, but finally had comparable little effect on the overall energy yields per hectare.

From the figures shown it is clear, that a final evaluation of a crop to biogas system needs a careful investigation of the local growth conditions for specific plants. Generalised forecasts are not possible. Seasonal variations can completely change the picture and local framework conditions can considerably influence the results.

4.3.10 Energy requirement of crop biomethanation

Energy is required for crop production and as process energy in the biomethanation process. Furthermore energy is required for digestate transport- or upgrading. Energy transfer losses occur during utilisation of the biogas in engines, boilers, or during upgrading of biogas. As a very simplified, rough estimation, a 15 % process energy demand for the digestion process (Neubarth et al. 2000) is calculated for the following considerations.

In crop production energy is required for ploughing, seedbed cultivation, fertilising, pesticide- and herbicide application, harvest and transport. Furthermore considerable energy is required for the production of fertilisers, pesticides and herbicides. Detailed estimations exist for the various expenditures and can be used for the calculation of net energy production. The total energy input is highest for potatoes (24.2 GJ/ha) and beets (16.8-23.9 GJ/ha), wheat, barley and maize consume between 14.5-19.1 GJ/ha (Refsgaard et al., 1998; Hülsbergen and Kalk, 2001; Tzilivakis et al. 2005). In average, about 50 % of the total energy requirement is spent for fertiliser production, minor amounts are required for machinery (22%), transport fuel (15%) and pesticides (13%). These figures may vary in a fairly wide range, depending on local soil- and climate conditions during cropping.

For a rough calculation of the net energy yield in energy crop digestion, the mean values for potatoes, maize, fodder beet, oilseed rape and rye are taken as examples (Table 13.12). These crops cover the whole range of highest (about 10,000 $m^3.ha^{-1}$ methane) to lowest (about 1,000 $m^3.ha^{-1}$ methane) calculable yields.

Table 13.12. Simplified calculation of net energy yield and output / input ratios, for a wide range of calculable methane yields per ha, respectively selected examples of crops.

	Potatoes	Maize	Fodder beet	Oilseed rape	Rye
Methane yield $m^3 . ha^{-1}$	10,258	9,886	9,450	1,442	814
$MJ . ha^{-1}$	367,236	353,919	338,310	51,623	29,141
Process energy demand for digestion	- 55,085	- 53,088	-50,746	- 7,745	- 4,371
Energy requirement in cropping	- 24,200	- 16,800	- 20,350	- 16,800	- 16,800
Total energy requirement	- 79,285	- 69,888	- 71,096	- 24,545	- 21,171
Net energy yield $MJ.ha^{-1}$	287,951	284,031	267,214	27,078	7,970
$\frac{\text{Output } (MJ.ha^{-1})}{\text{Input (tot. Energy)}}$	4.6	5.1	4.8	2.1	1.4

If the process energy demand (15%) and the energy requirement for crop production (16,800-24,200 $MJ.m^{-3}$) are subtracted from the primary methane yields, the respective net energy produced per hectare is obtained. As can be seen the net energy production varies in a broad range of 8-288 GJ per hectare. The respective energy input / output ratios vary between 1.4 (rye) in the worst case and 5.1 (maize) in the best case.

With the assumptions chosen, a net energy recovery can be achieved even in the worst case of poor crop yields (e.g. rye). But the overall economy and feasibility of a technical process application depends on numerous further influential parameters. The economic viability of energy crop digestion is indispensable dependent on a high return on energy sales. From practical experience an economic operation can only be achieved, if high crop and biogas yields can be realised at reasonable low production costs (Keymer and Schilcher 1999). Additional benefits i.e. gate fees for co-substrates, subsidised feed in power tariffs, may improve the overall economics substantially and have to be considered carefully for the process evaluation.

5. Future significance of biomass and biomethanation

Biomethanation is successfully used for the treatment of sewage sludge and solid organic waste fractions, as well as for animal manure-, industrial sludge-, industrial- and municipal waste-waters. Applications in waste treatment produce significant benefits beyond simple waste removal. These benefits include both, energy production and energy conservation. In addition to waste removal, other environmental benefits result from biomethanation, including odor reduction, pathogen control, minimizing sludge production, conservation of nutrients and reduction of greenhouse gas emissions. Unlike fossil fuels, use of renewable resources represents a closed carbon cycle and therefore does not contribute to increases in atmospheric concentrations of carbon dioxide. Replacement of fossil fuels also reduces atmospheric pollutants responsible for acid rain. Thus, technical applications of methanogenesis are both, a waste treatment technology, which enhances environmental quality and a sustainable renewable energy-producing technology. Based on energy costs, biogas production from waste alone, may not always prove economically viable. Yet, applications of the process may produce significant energy savings and the benefits of energy conservation may also have significant economic value. Furthermore biomethanation can advantageously be integrated in a cascade type of raw material utilisation. By-products from biotechnological processes, i.e. slops, spent liquors, press cakes, can be further used for energy recovery. Biomass based "Biorefinery" concepts for biochemical production frequently include biomethanation processes for closing nutrient cycles and energy recovery (Sanders 2005).

Biomethanation is facing a big challenge in renewable energy generation from crops. A wide range of materials can be principally used to provide energy. Once it is dry enough, anything organic can be burnt directly or used in thermal gasification processes. On the other hand, oil crops can be directly used for fuel generation. Green plants frequently contain considerable high amounts of water and, as a source for energy generation, in most cases are preferably used indirectly, in bioconversion processes for ethanol or methane generation. While combustion- and thermal gasification processes achieve overall energy efficiencies of 70-80%, bioconversion processes range from about 30% (e.g. ethanol fermentation) to about 50% for biomethanation (Kleemann and Meliß 1988). Thermal processes destroy the structure of the organic substance, finally resulting in inorganic ash, while bioconversions protect valuable organic structures and residues can be advantageously applied as fertiliser or soil conditioner. Finding processes and crops yielding the best "value" in terms of energy production, while causing the least environmental drawbacks, still is a major

challenge of bio-energy generation. Furthermore a cascade use of biomass, aiming at high value products primarily, must be realised.

Soil principally has to serve for numerous functions, of which biomass production is one of the most important. Biomass is providing food and fodder, it is used for numerous technical purposes and last not least can serve as an energy source. Since cropping areas and forests are limited in many regions, different biomass uses often are in direct competition in land use. Terestrial biomass principally can be obtained from forestry and short rotation forestry (e.g. willow, poplar, eucalyptus), from herbaceous lignocellulosic crops (e.g. Miscanthus), from sugar- (e.g. sugar beet, sweet sorghum, Jerusalem artichoke) and starch crops (e.g. maize, wheat), from oil crops (e.g. rape seed, sunflower) and from different kinds of wastes. The worldwide existing biomass is estimated to be 2.10^{12} tons (Kaltschmitt and Hartmann 2001). The annual biomass growth is about $1.55.10^{11}$ tons, corresponding to 3.10^{21} J. It is estimated that only a minor amount of the annual biomass growth is really used actually for food and fodder (2%), technical purposes (1%) and energy generation (1%). Using the above figures, an easy calculation shows, that about 13% of the annual biomass growth ($1.55.10^{11}$ tons), could theoretically supply the worldwide overall primary energy demand of $3.95.10^{20}$J. The estimations for the technically, respectively economically feasible contribution differ widely. Hall et al. (1993) have estimated, that about ⅔ (267 EJ) of the worldwide overall primary energy demand could be provided from biomass. Kaltschmitt and Hartmann (2001) state a potential biomass contribution of 29% to the worldwide- and 12% to the European primary energy demand. According to the above authors, dedicated energy crops for biomethanation could contribute 10% to the worldwide- and 3.5% to the European primary energy demand.

The growing interest in bio-energy is reflected in a large number of energy scenarios, published in the past decade. Numerous institutions have tried long term prognosis and studies of future scenarios (Dessus et al. 1992; Greenpeace 1993; IEA 1998; IIASA/WEC 1998; IPCC 1996; Johansson et al. 1993; Lashof and Tirpak 1991; Shell 1996; WEC 1993). In the scenarios biomass contribution is estimated in the range of 59-135 EJ/year in 2025, 94-220 EJ/year in 2050 and 132-320 EJ/year in 2100. Based on the current contribution of biomass (55 EJ/year) the figures given imply an increase of biomass of 7-245% by 2025, 170-400% by 2050 and 240-581% by the year 2100.

The traditional role of agriculture in energy production was lost in the younger history, since biomass used to be the main source of energy up to the early 20th century. Most of other things needed daily, e.g. food, fodder, fertiliser, fibres etc., were also derived from biomass. The current contribution of biomass to energy generation is comparably small in most industrialised countries (3-5%), about 20-30% in Asian countries and

about 70-80% of the overall energy demand in Africa. With the exception of Finland (19.1%), Sweden (16.%), Portugal (13.5%) and Austria (11.1%) biomass is rarely used as energy source in the European Union (Koukios 2002), resulting in an overall mean contribution of only 3.3%. Nevertheless the European Commission's "White Paper on Renewable Energy" envisages a doubling of renewable energy utilisation rates from 6% in 1995, to 12% by 2010. Biofuels are specially promoted by the Directive (EC) 30/2003, setting a goal of 5,75% diesel and petrol replacement by 2010. The "Biomass Action Plan" of the Commission of the European Communities (Anonym 2005a) estimates the current contribution of energy crops to the European overall primary energy demand (74.8 EJ/year) to be 1.12%. Predictions for 2010 are given with 25% and for 2020 with 47% contribution. In 2030, the generation of energy from crops could be even higher than the demand. The current energy recovery from biomass wastes is estimated to be 4.5%, predictions are about 56% for 2010 onwards.

The surface of the earth is mostly covered by oceans (361.10^6 km^2). From the remaining area of 149.10^6 km^2 about 55.7% are covered by forests, 16.1% (or 24.10^6 km^2) is pastureland and only about 9.4% (or 14.10^6 km^2) is arable land (Lieth 1975). Europe has a total area of $2.3.10^6$ km^2, of which 41% consist of forests, 13% of arable land and 8 % of pasture land.

Assuming a mean biogas net energy yield of 150 GJ / hectare (compare table 12), 10 % of the arable land would provide 21 EJ (worldwide), or 4.5 EJ (Europe) from biomethanation of crops. If energy crops could be cultivated on 30% of the existing arable land, about 16% (worldwide) or 18% (Europe) of the overall primary energy demand could be covered. Even using theoretically all of the available arable land, just about 53 % of the worldwide and 60% of the European energy demand would be satisfied through biomethanation of energy crops. A further biogas contribution could be expected from pasture land resources, although harvest may be more expensive and the overall energy yields may be less than from dedicated grown energy crops. As can be seen form the above figures, biomethanation can contribute substantially to the renewable energy supply, but cannot be considered as the main source of bio-energy.

Theoretically sufficient agricultural areas and forests are available to scope with an increasing demand of bio-energy. Nevertheless numerous technical-, environmental- and economical restrictions have to be kept in mind. Adverse effects of increased energy cropping on food production (competition), biodiversity, soil and groundwater must be prevented. Pests and diseases, respectively intensive monocultures, must be prevented. A shift towards a more environmentally friendly farming should not be adversely affected. Land use and forestry should follow sustainable patterns and permanent grass land should not be transformed into forests

excessively. Energy crops principally should not displace land uses of high agricultural and ecological value. Harvesting operations, transport methods and distances to the conversion plants significantly impact the overall energy balance of a biomass to energy system. Careful evaluation is therefore required to minimise transport energy demand for low energy density biomass, as well as adverse affects on air, water and soil.

Provided that the majority of the biomass needed additionally can be produced locally, a considerable impact of bioenergy exploitation on rural employment can be expected. The EU "Biomass Action Plan" assumes that direct employment of up to 300,000 people, mostly in rural areas, will be needed additionally to scope with the employment intensive energetic biomass use. Since biomethanation of crops not only delivers directly usable energy, but also fits excellently in a sustainable agricultural nutrient cycle, the process will receive increasing interest, among other renewable energies available.

Recent developments in Germany, Sweden, Denmark and Austria have proved the possibility of implementing biogas technology in rural infrastructure. The number of agricultural biogas plants in Germany has increased from about 100 in the early 1990s, up to more than 3,000 current installations (Weiland 2005). Assuming mean biogas yields, about 14 PJ / year can be obtained from these plants. For using the whole German potential of organic wastes and possible energy crops, an additional installation of 30,000-40,000 biogas plants would be required, resulting in a future energy potential of more than 400 PJ. In Sweden about 200 biogas plants produce 140.10^6 m^3 methane, corresponding to about 0.39 PJ /year. In Denmark 3.6 PJ / year are obtained from over 30 large scale, centralised agricultural biogas plants. About 40 new plants are expected until 2008 (Al Seadi 2005). In Austria the number of biogas plants was increasing within short time from about 50 to about 350 plants in 2006, representing an energy production of about 2 PJ per year. Accordingly, 3,000-4,000 new biogas plants can be estimated to be required for the use of proper substrates available in the nearer future. Movements towards increased biogas technology application can also be observed in some other European countries, e.g. Italy, Spain, Netherlands. Although the major sources of biogas in many countries, like England or France, still are landfills and sewage, the use of crop materials may become more important with gradually improved framework conditions.

References

Al Seadi T (2005) Country report Denmark. IEA Bioenergy Task 37, Energy from Biogas & Landfill Gas. International Energy Agency, Paris, France

Amon T, Kryvoruchko V, Amon B, Moitzi G, Buga S, Fistarol Lyson D, Hackl E. and Jeremic D (2003) Biogas production from the energy crops maize and clover grass. Final Report No. 1249 GZ 24.002/59-IIA1/01 to the Austrian Federal Ministry of Agriculture & Environment. Univ. of Nat. Res. & Appl. Life Sciences, Vienna, Austria

Amon T, Kryvoruchko V, Bodiroza V, Amon B (2005) Methane production from cereals, grass and sun flowers: effect of harvesting time, and pre-treatment on the methane yield. In: KTBL: 7. Internationale Tagung Bau, Technik und Umwelt in der Nutztierhaltung. Braunschweig, Germany

Anderson GK Duarte A and Donelly T (1981) Application of the anaerobic contact process to industrial waste treatment. 34th Int. Symp. CEBEDEAU, Liege, Belgium

Andrews JF (1969) Dynamic model of the anaerobic digestion process. J. Sanit. Eng. Div. Proc. Amer. Soc. Civ. Eng. 95: 95-116

Angelidaki I, Ellegaard L and Ahring BK (2003) Application of the anaerobic digestion process. In: Ahring BK (ed) Biomethanation II, Springer

Anonym (1997) Systems and Markets Overview of Anaerobic Digestion. IEA Bioenergy Anaerobic Digestion Activity Brochure 1997. Lusk P (ed) Resource Dev. Assoc., 240 Ninth Street, NE, Washington D.C. 20002-6110, USA

Anonym (2001) Eurostat environment statistics, Data 1980-1999 ; OPOCE, Luxembourg.

Anonym (2001) European Guideline on “Renewable Energies” (EC) 77/2001

Anonym (2002) “Ökostromgesetz”. Austrian BGBl. II Nr. 508/2002

Anonym (2004) Biogas Energy Barometer. Eurobserver August 2004, 69-78

Anonym (2005a) Biomass action plan. COM (2005) 628 final. Commission of the European Communities, Brussels, Belgium

Anonym (2005b) Integrated pollution prevention and Control – Reference document on best available technology for the waste treatment industries. European Commission, Directorate General CRC, Institute for prospective Technological Studies, Sevilla, Spain

Anonym (2005c) Statistics Handbook Austria 2005. Statistik Austria; Vienna, Austria

APHA (1998) Standard methods for the examination of water and wastewater. American Public Health Association, Washington D.C., USA

Atzema AJ (2005) Practical experience of the cultivation of Red Canary Grass, Switchgrass and Miscanthus in the northeast of the Netherlands. Paper presented at International Energy farming Congress, Papenburg, Germany

Austermann-Haun U, Wendler D and Rosenwinkel KH (2001) Großtechnische Erfahrungen mit der Co-Fermentation in Deutschland. KA-Wasserwirtschaft, Abwasser, Abfall 48 (10): 1443-1451

Baader W (1983) Technische Aspekte der Biogaserzeugung aus landwirtschaftlichen Abfall- und Reststoffen. In: Fortschritte beim Biogas. Kuratorium für Technik und Bauwesen in der Landwirtschaft, Darmstadt, Germany, KTBL 285, 160-165

Badger DM, Bogue MJ and Stewart DJ (1979) Biogas production from crops and organic wastes: 1.- Results of batch digestions. New Zealand Journal of Science 22: 11-20

Barker HA (1956) Bacterial fermentations. N.Y. John Wiley & Sons, New York

Bechamp A (1868) Lettre a M. Dumas. Ann. Chim. Phys. Ser. 4, 13: 103-111

van den Berg L, Kennedy KJ and Hamoda MF (1981) Effect of type of waste on performance of anaerobic fixed film and upflow sludge bed reactors. 36th Industrial Waste Conference, Purdue Univ., Lafayette, Ind., USA

Becker KP (1984) Biologische Abwasserreinigung im Fließbettverfahren, dem ANITRON System, dem OXITRON System sowie im Membran Anaerob Reaktor System MARS. Neueste Verfahrenstechnologien in der Abwasserreinigung, Abwasser- und Gewässerhygiene. R. Oldenbourg, München, Germany

Becker KP (1983) The membrane anaerobic reactor system (MARS) for efficient treatment of ultrahigh polluted industrial wastewaters. Proceedings of the European Symposium AWWT, Noordwijkerhout, The Netherlands, p 294-295

Bendixen HJ (1999) Hygienic safety – results of scientific investigations in Denmark (Sanitation requirements in Danish biogas plants). In: Böhm R and Wellinger A (eds) IEA Bioenergy Workshop Vol. 2, Deutsche veterinärmedizinische Gesellschaft e.V., Gießen, Germany, pp 27-47

Bendixen HJ and Amendrup S (1992) Safeguards against pathogens in biogas plants. Practical measures to prevent dissemination of pathogens and requirements for sanitation. Report of the Danish Veterinary Service, Copenhagen, Denmark

Black MG, Brown JM and Kaye E (1974) Operational experience with an abattoir waste digestion plant at Leeds. Water Poll. Control 78 (5): 532-537

BMWA (2003) Technische Grundlage für die Beurteilung von Biogasanlagen. Arbeitskreis Biogasanlagen, BMWA – Federal Ministry of Trade and Labour, Wien, Austria

Boe K, Batstone DJ and Angelidaki I (2005) Online headspace chromatographic method for measuring VFA in biogas reactor. Water Science & Technology 52 (1-2): 473-478

Böhnke B, Bischofsberger W and Seyfried CF (1993) Anaerobtechnik – Handbuch der anaeroben Behandlung von Abwasser und Schlamm. Springer, Berlin, Germany

Borghans L (1987) Anaerobic waste treatment in the ethanol industry. Fuel Ethanol Workshop, Minneapolis, Minnesota, USA

Böhnke B, Bischofsberger, W and Seyfried CF (1993) Anaerobtechnik Handbuch der anaeroben Behnadlung von Abwasser und Schlamm. Springer, Berlin, Germany

Braun R, Huber P and Meyrath J (1981) Ammonia toxicity in liquid piggery manure digestion. Biotechn. Letters 3: 159-164

Braun R and Huss S (1982) Anaerobic filter treatment of molasses distillery slops. Water Research 16: 1167-1171

Braun R (1982) Biogas –Methangärung organischer Abfallstoffe. Springer, Berlin, Germany

Braun R and Kirchmayr R (2004) Legislative aspects. In: Lens P, Hamerlers B, Hoitink H and Bidlingmaier W (eds) Resource recovery and reuse in organic solid waste management. IWA Publishing, London, UK

Bro B (2000) Grindsted – Codigestion of Sewage Sludge and Household Waste. Conference „Kick-off for a Future Development of Biogas Technology", Eskilstuna, Sweden

Brondeau P and Girardon PH (1982) Procede Biopural traitement par filtre anaerobic des effluents liquids del lìndustrie agro-alimentaire et de lèvage. In: Strab A, Cartier P and Schleser G (eds) Energy from biomass, 2nd EC Conference. Applied Science Publishers, London – New York

Bryant MP (1974) Methane producing bacteria. In: Buchanan &Gibbons (eds) Manual of determinitive bacteriology, 8th Ed. Baltimore, Md., The William & Wilkins Company.

Bryant MP (1977) The microbiology of anaerobic degradation and methanogenesis with special reference to sewage. In: Schlegel HG, Barnea J (eds) Microbial energy conversion. Pergamon Press, Oxford

Carrington EG (2001) Evaluation of sludge treatments for pathogen reduction – final report. Office for official publications of the European Union, Luxemburg.

Carrington EG, Pike EB, Auty D and Morris R (1991) Destruction of faecal bacteria, enteroviruses and ova of parasites in wastewater sludge by aerobic thermophilic and anaerobic mesophilic digestion. Water Sci. Tech. 24 (2): 377-380

Checchi F, Traverso PG, Mata-Alvarez J, Clancy J and Zaror C (1988) State of the art of R&D in the anaerobic digestion process of municipal solid waste in Europe. Biomass 16: 257-284

Chen Ruchen (1981) The development of biogas utilisation in China. Biomass 1 (1): 39

Cheremisionoff PN and Moressi AC (1976) Energy from solid wastes. Marcel Decker, New York, USA

Chin KK and Goh TN (1978) Bioconversion of solar energy: Methane production through water hyacinth. Proceedings of the 2nd Symposium on Energy from Biomass and Wastes, Inst. of Gas Technology, Chicago, USA, pp 215-228

Christensen DR, Gerick JA and Eblen JE (1984) Design and operation of an anaerobic upflow sludge blanket reactor. IWPCF 56(9) 1059-1062

Cillie GG, Henzen MR, Stander GJ and Baillie RD (1969) Anaerobic digestion. IV. The application of the process in waste purification. Water Res. 3: 623-643

Coulter JB, Soneda S and Ettinger MB (1957) Anaerobic contact process for sewage disposal. Sew. Ind. Wastes 29: 468-477

Dauber S (1993) Anaerobtechnik – Handbuch der anaeroben Behandlung von Abwasser und Schlamm. Springer, Berlin, Germany

DeBaere L, Verdonck O and Verstraete W (1985) High rate anaerobic composting process for the organic fraction of solid wastes. Biotechnology & Bioengineering Symposium 15: 321-330

DeBaere L (1999) Anaerobic digestion of solid waste state of the art. 2nd Int. Symp. Anaerobic Digestion of Solid Waste, Barcelona, Spain

DeBaere L (2005) Will anaerobic digestion of solid waste survive the future? In: Ahring B and Hartmann H (eds) Proceedings of the 4th Int. Symposium Anaerobic Digestion of Solid Waste. Techn. Univ. Denmark, Copenhagen

Degenhart H (2005) Optimierung des Biogasertrages durch angepasste Maissorten und richtiges Anbaumanagement. Paper presented at International Energy farming Congress. Papenburg, Germany

Demuynck M, Nyns EJ and Palz W (1984) Biogas Plants in Europe. Reidel Publ., Dordrecht

Dessus B, Devin B and Pharbod F (1992) World potential of renewable energies. La Hille Blanc 1: 1-50

DeRenzo DJ (1977) Energy from bioconversion of waste materials. Noyes Data Corporation, Park Ridge, New York

DEV (2005) Deutsche Einheitsverfahren zur Wasser-, Abwasser- und Schlamm-Untersuchung – Physikalische, chemische, biologische und bakteriologische Verfahren. Wiley-VCH, Weinheim, Germany

Diaz LF, Savage GM and Golueke EG (1982) Resource recovery from municipal solid waste. Vol. 2, CRC press, Boca Raton, Fla., USA

Dichtl N and Sixt H. (1996) Faulgas- / Biogaserzeugung. In: ATV Handbuch Klärschlamm. Abwassertechn. Vereinigung (Hrsg.) Verlag Ernst & Sohn, Berlin, Germany

Donnely T (1978) Industrial effluent treatment with the bioenergy process. Process Biochem. 13: 14-16

Ebeling M (2005) Biogasproduktion im Ökobetrieb – Praxiserfahrungen. Paper presented at International Energy farming Congress, Papenburg, Germany

Eckenfelder WW and O`Connor DJ (1961) Biological waste treatment. Pergamon Press, Oxford, UK

Edelmann W (2001) Biogaserzeugung und –nutzung. In: Kaltschmitt M and Hartmann H (eds) Energie aus Biomasse – Grundlagen, Technik und Verfahren.; Springer, Berlin, Germany

Eder B (2005) Anbau, Düngung und Biogaserträge von nachwachsenden Rohstoffen. Paper presented at International Energy farming Congress, Papenburg, Germany

Ehhalt DH (1974) The atmospheric cycle of methane. In: Schlegel HG, Gottschalk G and Pfenning N (eds) Microbial production and utilisation of gases. Akademie der Wissenschaften, Göttingen, Germany

Fannin KF, Srivastava VJ and Chynoweth DP (1982) Unconventional anaerobic digester design for improving methane yields from sea kelp. Proceedings of the 6th Symposium on Energy from Biomass and Wastes. Inst. of Gas Technology, Chicago, pp 373-399

Finney CD and Evans RS (1975) Anaerobic digestion – the rate limiting process and the nature of inhibition. Science 190: 1088

Fischer T and Krieg A (2005) Monofermentation von Energiepflanzen – Erfahrungen von der Biogasanlage Obernjesa. VDI Berichte 1872, 153-163

FNR (2005) Handreichung Biogasgewinnung und –nutzung. Fachagentur Nachwachsende Rohstoffe e.V., Gültzow

Gallert C, Henning AK, Stentzel U and Winter J (2002) Verarbeitung von getrennt gesammelten Bioabfällen in der Bioabfall Vergärungsanlage Karlsruhe Durlach. KA Wasserwirtschaft, Abwasser, Abfall 49 (5): 695-704

Gessler G and Keller K (1995) Vergleich verschiedener Verfahren zur Vergärung von Bioabfall. Abfallwirtschaftsjournal 7 (6): 377-382

Grady CPL jr. and Lim HC (1980) Biological wastewater treatment. Marcel Decker, New York, USA

Greenpeace (1993) Towards a fossil free energy future: The next energy transition. A technical analysis for Greenpeace International by the Stockholm Environment Institute. Boston Centre, Boston, USA

Gunaseelan VN (2004) Biochemical methane potential of fruits and vegetable solid waste feedstocks. Biomass & Bioenergy 26 (4): 389-399

Hall DO, Rosillo-Calle F, Williams RH and Woods J (1993) Biomass for Energy: Supply Prospects. In: Johannson BJ (ed) Renewables for fuels and electricity. Island Press, Washington DC, USA

Heertjes PM and van der Meer RR (1978) Daynamics of liquid flow in upflow reactor used for anaerobic treatment of wastewater. Biotechn. Bioeng. 20: 1577-1594

Heiermann M, Plöchl M, Linke B and Schelle H (2002) Preliminary evaluation of some cereals as energy crops for biogas production. In: Sayigh AAM (ed) Proceedings World Renewable Energy Congress VII. Elsevier Sci. Ltd

Heiermann M and Plöchl M (2004) Crops – A big potential for biogas production. Proceedings of the World Renewable Energy Production Congress VIII. Denver, Colorado, USA

Heijnen JJ, Enger WA, Moulder A, Lourens PA, Keijzers AA and Hoeks FWJMM (1985) Anwendung der anaeroben Wirbelschichttechnik in der biologischen Abwasserreinigung. Gwf – Wasser / Abwasser 126 (2): 81-87

Henderson C, (1973) The effect of fatty acids on pure cultures of rumen bacteria. J. Agric. Sci. 81: 107

Hobson PN, Bousfield S and Summers R (1981) Methane production from agricultural and domestic wastes. Applied Science Publishers, London, UK

Hoppe W (1956) Entwicklung und Stand der Klärgasgewinnung und Klärgasverwertung in Deutschland. In: Liebmann H (ed) Gewinnung und Verwertung von Methan aus Klärschlamm und Mist. R. Oldenbourg, München, Germany.

Hoppenheidt K and Mücke W (1999) Gemeinsame Behandlung von Bioabfällen und organischen Gewerbeabfällen durch Co-Vergärung. Bayerisches Landeamt für Umweltschutz, BlfA Texte No. 11, München, Germany

Hulshoff Pol LW, De Zeeuw, W, Dolfing J and Lettinga G (1983) Start up and sludge granulation in the UASB reactors. Proceedings of the European Symposium AWWT, Noordwijkerhout, The Netherlands

Hülsbergen KJ and Kalk WD (2001) Energy balances in different agricultural systems – Can they be improved? Proceedings of the International Fertiliser Siciety No. 476, The International Fertiliser Society, York, YO32 5YS, UK

IEA (1998) World energy outlook. International Energy Agency. Paris, France

IIASA/WEC(1998) Global energy perspectives. Nakicenovic N (ed), Cambridge Univ. Press, Cambridge, UK

IPCC (1996) Climate change 1995: Impacts, adaptations and mitigation of climate change: Scientific and technical analysis. Intergovernmental Panel on Climate Change, Working Group II Report. Cambridge Univ. Press, Cambridge, UK

Johansson TBJ, Kelly H, Reddy AKN and Williams RH (1993) Renewable fuels and electricity for a growing world economy. In: Johansson TBJ (ed.) Renewables for fuels and electricity. Island Press, Washington, DC, USA

Jönsson O (2005) Country report Sweden 2005. IEA Bioenergy Task 37 "Energy from Biogas & Landfill Gas". Int. Energy Agency, Paris, France

Kaiser F (2004) Methanerträge verschiedener nachwachsender Rohstoffe. Biogas Journal (2): 22-24

Kaltschmitt M and Hartmann H (2001) Energie aus Biomasse. Springer, Berlin, New York

Kaparaju P, Luostarinen S, Kalmari E, Kalmari J and Rintala J (2002) Co-digestion of energy crops and industrial confectionary by-products with cow manure: Batch scale and farm scale evaluation. Water Sci. & Technol. 45 (19): 275-280

Kearney TE, Larkin MJ, Frost JP and Levett PN (1993) Survival of pathogenic bacteria during mesophilic anaerobic digestion of animal waste. J. Appl. Bacteriol. 75: 215 – 219

Keimer U, and Schilcher A (1999) Ist die Vergärung von Silomais in einer Biogasanlage sinnvoll? Biogas Journal (3) 10-12

Kemmer FN (1979) The NALCO water handbook. McGraw-Hill, New York, USA

Keppler F, Hamilton JTG, Braß M and Röckmann T (2006): Nature 439,:187-191

Kern M (1999) Stand und Perspektiven der biologischen Abfallbehandlung in Deutschland. In: Wiemer K and Kern M (eds) Bio- und Restabfallbehandlung III. S 293-321, Baeza, Witzenhausen, Germany

Kirchmayr R, Scherzer R, Baggesen DL, Braun R and Wellinger A (2004) Animal by-products and anaerobic digestion, requirements of the European Regulation (EC) No. 1774/2002. Brochure IEA Bioenergy Task 37 – Energy from biogas and landfill gas. Nova Energie, Aadorf, Switzerland

Kirchmayr R, Braun R and Baumann F (2000) Anaerobe Verwertung von Schlachtnebenprodukten. Die Fleischmehlindustrie 52 (12): 231-235

Klass L (1998) Biomass for renewable energy, fuels and chemicals. Academic Press, San Diego, Ca., USA

Kleemann M and Meliß M (1988) Regenerative Energiequellen. Springer, Berlin New York

Klink G (2005) Endproduktaufreinigung. Paper presented at Symposium "Erfahrungsaustausch für Betreiber von Kompostierungs- und Biogasanlagen. Schloß Wolfpassing, Steinakirchen am Forst, Austria

Kloss R (1986) Planung von Biogasanlagen nach technisch – wirtschaftlichen Überlegungen. R. Oldenbourg, München, Germany

Kolb B (1986) Head Space Gaschromatographie mit Kapillar Trennsäulen. Vogel, Würzburg, Germany

Koukios EG (2002) Biomass. In: Eurec Agency (ed) The Future for Renewable Energy 2 – Prospects and Directions., James & James Science Publ., London, UK

Koot ACJ (1980) Behandling van Afalwater. Waltman, Delft, Holland

Korz DJ (1999a) Naßvergärungsanlagen in Deutschland. Entsorgungspraxis 3: 39-41

Korz DJ (1999b) Vergärung von Hausmüll auf Sardinien. Entsorgungspraxis 7-8: 29

Koyama T (1963) Gaseous metabolism in sediments and paddy soils and the production of atmospheric methane and hydrogen. J. of Geophys. Res. 68: 3971-3973

Krull R, Diekmann R and Lindert M (1995) Behandlung von Bioabfall in Vergärungsanlagen – eine Marktübersicht. Entsorgungspraxis 5: 22-25

Kuhn E (1995) Kofermentation. Kuratorium für Technik und Bauwesen in der Landwirtschaft e.V. Darmstadt, Germany. KTBL, Arbeitspapier 219

Laaber M, Kirchmayr R, Madlener R and Braun R (2005) Development of an evaluation system for biogas plants. 4th Int. Symposium Anaerobic Digestion of Solid Waste. Copenhagen, Denmark

Landbeck M (2005) Energiemais, Ziele, Strategien und erste Züchtungserfolge. Paper presented at International Energy farming Congress. Papenburg, Germany

Landine RC, Brown JG, Cocci AA and Viraraghavan T (1980) Potato processing wastewater treatment using hozontal anaerobic filters. Can. Inst. Food Sci. Technol. Journal 14(2): 144-146

Langhans G (2000) Co-Vergärung in der Praxis. Abfall Brief – Magazin zur Abfallpraxis 7(2): 7-9

Lashoff DA and Tirpak DA (1991) Policy options for stbilising global climate. Report to the Congress, Technical Appendices. Office of Policy, Planning and Evaluation; US Environmental Protection Agency, Washington, DC., USA

Lawrence AW and McCarty PL (1964) Effects of sulphide on anaerobic treatment. Proceedings of the 19th Purdue Industrial Waste Conference. Purdue Univ., Ann Arbor, Mich., Ann Arbor Science

Lähtomäki A Viinkainen TA Alen R and Rintala JA (2003) Methane production from energy crops and crop residues: Effect of harvest time and chemical composition. Proceedings of the International Nordic Bio-energy Conference. Jyväskylä, Finland

Lähtomäki A Ronkainen OM and Rintala JA (2005) Developing storage methods for optimised methane production from energy crops in northern conditions. In: Ahring BK and Hartmann H (eds) Proceedings of the 4th Int. Symposium on Anaerobic Digestion of Solid Waste. Copenhagen, Denmark

Leschber R and Loll U (1996) ATV Handbuch Klärschlamm. Ernst & Sohn, Berlin, Germany

Lettinga G and Hulshoff Pol L (1988) Die Anwendung von Schlammbettreaktoren. Proceedings Symposium Verfahrenstechnik in der mechanischen-, thermischen-, chemischen- und biologischen Abwasserreinigung. Baden Baden, Germany, p 187-202

Lettinga G, van der Saar J. and van der Ben J. (1976) Anaerobe zuivering van het afalwater van de bietsuikerindustrie. H_2O 9, 38-43

Lettinga G, van Velsen L, de Zeeuw W and Homba S.W (1980) The application of anaerobic digestion to industrial pollution treatment. In: Wheatley DA and Hughes DE (eds) (1979) Proceedings of the 1st Int. Symp. Anaerobic Digestion. Applied Science Publishers, London, UK

Liebich T (2004) Minimierung von Konfliktpotenzial durch Gerüche an Biogasanlagen. 13th Annual Meeting of the German Biogas Association. Leipzig, Germany

Liebmann H (1956) Gewinnung und Verwertung von Methan aus Klärschlamm und Mist,. R. Oldenbourg, München, Germany

Lieth H and Whittaker RW (1975) The primary production of the biosphere. Springer, Berlin, Germany

Lindorfer H (2003) Vergleich von Meßmethoden zur Bestimmung der Konzentration flüchtiger Fettsäuren im Faulschlamm aus Biogasanlagen. Thesis, Univ. Natural Res. & Applied Life Sciences, Vienna, Austria

Lindorfer H, Kirchmayr R and Braun R (2005a) Self heating of anaerobic digesters using energy crops. In: Ahring BK and Hartmann H (eds) Proceedings of the 4th Int. Symposium Anaerobic Digestion of Solid Waste, International Water Association, London, UK

Lindorfer H (2005b) Massenbilanz einer 500 kW Energiepflanzenvergärungsanlage. Unpublished internal report of energy crop digester monitoring. Austrian Renewable Energy Network (RENET). A Research project sponsored by the Federal Ministry of Trade & Industry (BMWA), Wien, Austria

Linke B, Baganz K and Schlauderer R (1999) Nutzung von Feldfrüchten zur Biogasgewinnung. Agrartechnische Forschung 5(2): 81-90

Liqudara MJ and Ott ChR (1983) The treatment of apple processing wastewater by the anaerobic filter process. Proceedings of the 38th Ind. Waste Conference, Ann Arbor Science, Mich. USA

McBride BC and Wolfe RS (1971) Biosynthesis of dimethylarsine by *Methanobacterium*. Biochemistry 10, 23: 4312

McCarty PL (1982) History and overview on anaerobic digestion. In: Proceedings of the 2nd Int. Symposium Anaerobic Digestion Travemünde (1981). Elsevier Biomedical Press, Amsterdam, Holland

McCarty PL and McKinney RE (1961) Salt toxicity in anaerobic digestion. JWPCF 33, 399-415

McDonald P (1981) The biochemistry of silage. John Wiley & Sons, Chichester – New York – Brisbane – Toronto

McInerney MJ and Bryant MP (1980) Anaerobic degradation of lactate by syntropic associations of *Methanosarcina barkeri* and *Desolfovibrio sp.* and effect of H_2 on acetate degradation. Appl. Env. Microbiol. 41: 346-454

Mah RA (1977) Acetate a key intermediate in methanogenesis. In: Schlegel HG, Barnea J (eds) Microbial energy conversion. Pergamon Press, Oxford.

Mata-Alvarez J (2003) Biomethanization of the organic fraction of municipal solid wastes. IWA Publishing, London, UK

Märkel H and Stegmann R (1994) Anaerobe Behandlung von festen und flüssigen Rückständen. Dechema Monographien Volume 130, Dechema, Frankfurt/ Main, Germany

Martens W, Philipp W and Böhm R (2000) Seuchenhygienische Bewertung von Anaerobverfahren unter besonderer Berücksichtigung der landwirtschaftlichen Kofermentation. In: Wiemer K and Kern M (eds) Bio- und Restabfallbehandlung IV, Witzenhausen Institut für Abfall, Umwelt und Energie, Witzenhausen, Germany, pp 965-984

Mateu A, Mata-Alvarez J and Pares J (1992) Enterobacterial and viral decay experimental models for anaerobic degestion of piggery waste. Appl. Microbiol. Biotechnol. 38: 291-296

McKain N and Hobson PN (1987) A note on the destruction of porcine enteroviruses in anaerobic digestions. Biol. Wastes 22: 147-155

Melin T and Rautenbach R (2004) Membranverfahren. Springer, Berlin, Germany

Morgan PF and Neuspiel PJ (1958) Environmental control of anaerobic digestion with gas diffusion. In: McCabe J and Eckenfelder W (eds) Biological treatment of sewage and industrial waste. Reinhold, New York, N.Y., USA

Mosey FE, Swanwick JD and Hughes A (1971) Factors affecting the availability of heavy metals to inhibit anaerobic digestion. Water Poll. Control 6: 668-680

Müsken J and Bidlingmaier W (1994) Vergärung und Kompostierung von Bioabfällen. Texte und Berichte zur Abfallwirtschaft Volume 6, Landesanstalt für Umweltschutz, Karlsruhe, Germany

Neubarth J Mairitsch K Hofbauer H and Kaltschmitt M (2000) Biomasse. In: Neubarth J and Kaltschmitt M (eds) Erneuerbare Energien in Österreich. Springer, Wien New York

Neureiter M, Teixeira Pereira dos Santos J, Perez Lopez C, Pichler H, Kirchmayr R and Braun R (2005) Effect of silage preparation on methane yields from whole crop maize silages. In: Ahring BK and Hartmann H (eds) Proceedings of the 4th Int. Symposium on Anaerobic Digestion of Solid Waste. Copenhagen, Denmark.

Nordberg A (2005) Agrioptigas – Demonstration of an optimised production system for biogas from biological waste and agricultural feedstock. Paper presented at the Cropgen / IEA Bioenergy Task 37 Research Exchange Meeting, Utrecht, The Netherlands

Nyns EJ (1986) Biomethanation processes. In: Schönborn W (ed) VCH Weinheim Biotechnology Vol 8, Microbial degradations, pp 207-267

Oliva E Jaquart JC and Prevot C (1990) Treatment of waste water at the El Aquila Brewery Madrid, Spain. Methanisation in fluidised bed reactors. Water Sci. Techn. 22 (1/2): 483-490

Omelianski W (1906) Über Methanbildung in der Natur bei biologischen Prozessen. Zbl. Bakt. Parasitenkunde II, 15: 673-687

Pavan P, Battistoni P, Bolzonella D, Innocenti L, Traverso P and Checchi F (2000) Integration of wastewater and of MSW treatment cycles: From pilot scale to industrial realisation. The new full scale plant of Treviso. In: Hartmans S and Lens P (eds) Proceedings from the 4th Int. Symposium on Environmental Biotechnology, Noordwijkerhout, Holland

Pesaro F, Sorg I and Metzler A (1995) In situ inactivation of animal viruses and Coliphage in non-aerated liquid and semi-liquid animal wastes. Appl. Env. Microbiol. 61: 92-97

Philipp W and Böhm R (1997) Hygieneanforderungen an Verfahren der Boabfallvergärung. In: Wiemer K and Kern M (eds) Bio- und Restabfallvergärung pp 313-344, Witzenhausen Institut für Abfall, Umwelt und Energie; D-37213 Witzenhausen, Germany

Pette KC, de Vletter R Winds E and van Gils W (1980) Full scale anaerobic treatment of beet sugar waste water. Proceedings of the 35th Ind. Waste Conf. Purdue Univ., Purdue, Lafayette, Ind., Ann Arbor Science, USA

Pfeffer JT (1978) Methane from solid urban waste. The REFCOM project. Process Biochem. 13: 8

Pfeffer JT (1979) Anaerobic digestion processes. In: Stafford DA, Wheatley BI and Hughes, DE (eds) Proceedings of the 1st Int. Symposium on Anaerobic Digestion. Applied Science Publishers, London

Pipyn P, Verstraete W and Ombregt JP (1979) A pilot scale anaerobic upflow reactor treating distillery wastewaters. Biotechn. Letters 1: 495-500

Pohland, FG and Bloodgood DE (1963) Laboratory studies on mesophilic and thermophilic anaerobic sludge digestion. JWPCF 35, 11-42

Pouech P, Fruteau H, Bewa H (1998) Agricultural Crops for Biogas Production on Anaerobic Digestion Plants. Proceeding of the International Conference Biomass for Energy and Industry, Würzburg, Germany

Refsgaard K, Halberg N and Kristensen ES (1998): Energy utilisation in crop and dairy production in organic and conventional livestock production systems. Agriculturals Systems 57 (4): 599-630

Reinhold F and Noak W (1956) Laboratoriumsversuche über die Gasgewinnung aus landwirtschaftlichen Stoffen. In: Liebmann H (ed) Gewinnung und Verwertung von Methan aus Klärschlamm und Mist., R. Oldenbourg, München, Germany

Resch Ch. (2005) Massenbilanz einer 500 kW Energiepflanzenvergärungsanlage. Unpublished internal report of energy crop digester monitoring. Austrian Renewable Energy Network (RENET). A Research project of the Federal Ministry of Trade & Industry (BMWA), Wien, Austria

Roediger H (1967) Die anaerobe alkalische Schlammfaulung. R. Oldenbourg, München, Germany

Ross WE (1954) Dual Disposal of Garbage and Sewage at Richmond; Indiana, USA. Sewage & Industrial Waste 26: 2

Ruppel W, Biedron M, Thornton B and Swientek RJ (1982) Upflow anaerobic sludge blanket reduces COD 75-85%, produces methane gas. Food processing 10: 66-68

Ryther JH and Hasniak MD (1981) Anaerobic digestion and nutrient recycling of small benthic or floating seaweeds. Proceedings of the 5th Symposium on Energy from Biomass and Wastes, Inst. of Gas Technology, Chicago, pp 383-412

Sanders J (2005) Possibilities of integrating bio-energy (biogas) generation into other biomass based production systems. Paper presented at the Cropgen / IEA Bioenergy Task 37 Research Exchange Meeting, Utrecht, The Netherlands

Sanders FA and Bloodgood DE (1965) The effect of nitrogen to carbon ratio on anaerobic decomposition. JWPCF 37, 1741-1752

Seebaum H (1964) Trockenreinigung. In: Foerst W. (ed) Ullmanns Enzyklopädie der Technischen Chemie, Volume 15, Urban & Schwarzenberg, München-Berlin, Germany

Schink B (1992) Syntrophism among prokaryotes. In: Balows A, Trüper HG, Dworkin M, Harder W & Schleifer KH (eds) The Procaryotes, Springer Verlag, Berlin-Heidelberg-NewYork

Schmelz KG (2000) Energie aus Abfällen: Co-Vergärung in kommunalen Kläranlagen. Conference „Kommunale Chance: Energie aus Biomasse“, Inst. für kommunale Wirtschaft und Umweltplanung Gießen, Germany

Schön M (1994) Verfahren zur Vergärung organischer Rückstände in der Abfallwirtschaft. Erich Schmidt, Berlin, Germany

Schroepfer GJ, Fullen WJ, Johnson AS, Ziemke NR and Anderson JJ (1955) The anaerobic contact process as applied to packing house wastes. Sew. Ind. Wastes 27: 460-486

Shaw BG (1971): A practical and bacteriological study of the anaerobic digestion of waste from an intensive pig unit. PhD thesis, University Aberdeen, Scotland

Shell (1996) The evolution of the worlds energy system 1860-2010. Shell Int. Petroleum Co., London, UK

Silvers G (1991) Hygienisch–mikrobiologische Untersuchungen an der mesophil-thermophil arbeitenden Biogasanlage eines Milchviehbetriebes. PhD Thesis, Inst. für Umwelt- und Tierhygiene, University Stuttgart – Hohenheim, Germany

Speece RE (1996) Anaerobic biotechnology for industrial wastewaters. Archae Press, Nashville, Tennessee, USA

Spillmann SK, Traub F, Schwyzer M and Wyler R (1987) Inactivation of animal viruses during sewage sludge treatment. Appl. Environm. Microbiol. 53 (9): 2077-2081

Stafford DA, Hawkes DL and Horton R (1980) Methane production from waste organic matter. CRC Press, Boca Raton, Fla, USA

Stewart DJ (1980) Energy crops to methane. In: Stafford DA and Wheatley BI (eds) Anaerobic Digestion , Applied Science Publishers, London

Stewart DJ (1992) Energy from crop grown biomass. In: Baader W (ed) Biotechnologies for Pollution Control and Energies, FAL Technical Series 21, FAL, Braunschweig, Germany

Strauch D and Philipp W (2000) Hygieneaspekte der biologischen Abfallbehandlung und –verwertung. In: Bidlingmaier W (ed) Biologische Abfallverwertung, Eugen Ullmer GmbH & Co, Stuttgart, Germany, pp 155-208

Suidan MT, Strubler, CE, Kao SW and Pfeffer JT (1983) Treatment of coal gasification waste water with anaerobic filter technology. J. Water Poll. Control Fed. 55: 1263-1270

Summers R and Bousfield S (1980) A detailed study of piggery waste anaerobic digestion. Agr. Wastes 2: 61-78

Sykes RM and Kirsch EJ (1972) Accumulation of methanogenic substrates in CCl_4 inhibited sewage sludge digester cultures. Water Research 6: 41-55

TA – Luft (2002) Technische Anleitung zur Reinhaltung der Luft TA Luft (Germany); Ministerialblatt 30.6.2002 GMBl. 2002, Volume 25-29, pp 511-605 Internet: <http://www.bmu.de/files/taluft.pdf>

Taylor TW and Burn RJ (1973) Full scale anaerobic filter treatment on wheat starch plant wastes. Aiche Symp. Ser. 69, 30-37

Thomé-Kozmiensky KJ (1995) Biologische Abfallbehandlung. EF-Verlag für Energie- und Umwelttechnik, Berlin, Germany

Tidden F (2003) Leistunsgmerkmale und Grenzen der mehrphasigen anaeroben Behandlung kommunaler Bioabfälle. Berichte aus Wassergüte und Abfallwirtschaft der Technischen Universität München, Volume 176, München, Germany

Tzilivakis J, Warner DJ, May M, Levis KA and Jaggard K (2005) An assessment of the energy inputs and greenhouse gas emissions in sugar beet (Beta vulgaris) production in the UK. Agricultural Systems 85 (2): 101-119

Unterlerchner T (2006) Vergärung innovativer Substratkombinationen im Labormaßstab. Projektarbeit Fachhochschule Wiener Neustadt für Wirtschaft und Technik – Studiengang Biotechnische Verfahren Tulln, Austria

Van Doren HJ (2005) The use of grass from nature reserve areas as co-digestion material with cattle slurry. Paper presented at International Energy farming Congress. Papenburg, Germany

Van Velsen AFM (1979) Adaptation of methanogenic sludge to high ammonia nitrogen concentrations. Water Res. 13: 995-999

Verstraete W, DeBeer D, Pena M, Lettinga G and Lens P (1996) Anaerobic bioprocessing of organic wastes. World J. of Microbiol. & Biotechn. 12: 221-238

Vogels GD (1979) The global cycle of methane. Antonie van Leeuwenhoek 45: 347-352

Von Felde A (2005) Nawaro Biogasanlage ProN – Anlagenkonzeption und praktische Erfahrungen. Paper presented at International Energy farming Congress; Papenburg, Germany

WEC (1993) World Energy Council – Energy for tomorrows world. St. Martins Press, New York, USA

Weiland P (2003) Production and energetic use of biogas from energy crops and wastes in Germany. Applied Biochem. & Biotechnol. 109 (1-3): 263-274

Weiland P (2004) Erfahrungen deutscher Biogasanlagen – Ergebnisse einer bundesweiten Bewertung. Paper presented at the Minisymposium "Biogasgewinnung aus nachwachsenden Rohstoffen", University of Natural Resources & Applied Life Sciences Vienna, IFA Tulln, Austria

Weiland P (2005) Results and bottle necks of energy crop digestion plants - Required process technology innovations. Paper presented at the IEA Workshop "Energy Crops & Biogas", Utrecht, Holland

Wellinger A (2005) Country report Switzerland 2005. IEA Bioenergy Task 37 "Energy from Biogas & Landfill Gas". Int. Energy Agency, Paris, France

Wellinger, A, Edelmann W, Favre R, Seiler B und Woschitz D (1984) Biogas Handbuch. Wirz, Aarau, Switzerland

Wiemer K and Kern M (2000) Bio- und Restabfallbehandlung IV. Verlag Witzenhausen Institut für Abfall, Umwelt und Energie GmbH, Witzenhausen, Germany

Wiemer K and Kern M (1998) Kompost – Atlas 1998/99. M.I.C. Baeza, Witzenhausen, Germany

Wiemer K and Kern M. (1996) Biologische Abfallbehandlung III. M.I.C. Baeza, Witzenhausen, Germany

Winkelmann J (2005) Optimierung der Gasausbeuten bei Nawaros durch Siliermittel. Paper presented at the 14th Annual Meeting of IBBK „Biogas und Bioenergie in der Landwirtschaft". Kirchberg/Jagst Weckelweiler, Germany

Witt ER, Humphrey WJ and Roberts TE (1979) Full scale anaerobic filter treats high strength wastes. In: Ann Arbor, Mich Bell JM (ed) Proceedings of the 34th Ind. Waste Conf.; Purdue Univ., Ann Arbor Sci. Mich., USA

Yang PY (1981) Methane fermentation of Hawaiian seaweeds. Proceedings of the 5th Symposium on Energy from Biomass and Wastes, Inst. of Gas Technology, Chicago, pp 307-358

Young JC (1991) Factors affecting the design and performance of upflow anaerobic filters. Water Sci. Techn. 24 (8): 133-155

Young JC and McCarty PL (1969) The anaerobic filter for waste treatment. JWPCF 41, R160-R173

Zauner E, Küntzel U (1986) Methane production from ensiled plant material. Biomass 10, 207-223

Zeevalink JA and Maaskant W (1984) Biogas from effluent of starch industries by anaerobic treatment. Die Stärke 36, 131-135

Zubr J (1986) Methanogenic fermentation of fresh and ensiled plant materials. Biomass 11 (3): 159-171

Parameters, abbreviations and symbols

a	-	Year
BOD	[mg $O_2.l^{-1}$]	Biochemical oxygen demand
B_{TS}	[$kg.kg^{-1}.d^{-1}$]	Sludge loading rate
B_V	[$kg.m^{-3}.d^{-1}$]	Hydraulic or volumetric loading rate
COD	[mg $O_2.l^{-1}$]	Chemical oxygen demand
CHP	-	Combined heat and power plant
d	-	Day
$\Delta G_O^{`}$	[kJ/Mol]	Enthalpy
EJ	[10^{18} J]	Exajoule
GJ	[10^9 J]	Gigajoule
Methane number	-	Defines the pre-ignition resistance (knock rating) of a burnable gas
Mtoe	[10^7 Gcal]	Million tons of oil equivalent
Nm^3	-	Volume at standard conditions of 0°C and 101.325 kPa
NMHC	-	Non methane hydrocarbons
Odor units	-	That amount of odorant(s) that, when evaporated into one cubic metre of neutral gas at standard conditions, elicits a physiological response from a panel (detection threshold) equivalent to that elicited by one European Reference Odor Mass (EROM), evaporated in one cubic meter of neutral gas at standard conditions. [CEN TC264 Draft]
Pa	[1 N/m^2]	Pascal (1 bar = 10^5 Pa)
PJ	[10^{15} J]	Petajoule
ppm		Parts per million
θ	[d]	Hydraulic residence time
TJ	[10^{12} J]	Terajoule
TS	[%]	Total solids
VS	[%]	Volatile solids
v / v	[%]	Percent referred to volume
Wobbe index	[$MJ.m^{-3}$]	Amount of energy introduced to the burner
w / w	[%]	Percent referred to weight

14

Molecular farming for antigen (vaccine) production in plants

Chiara Lico, Selene Baschieri, Carla Marusic, Eugenio Benvenuto*

ENEA, C.R. Casaccia, Via Anguillarese 301, 00060 Roma, Italy (*e-mail: benvenutoe@casaccia.enea.it)

1 Molecular Farming and the new frontiers of vaccinology

Genomic and proteomic approaches to the study of fundamental cell mechanisms are rapidly contributing to broaden our knowledge on metabolic pathways for the optimal exploitation of the cell as a factory. In the last few years this knowledge has led to important advances in the large scale production of diagnostic and therapeutic proteins in heterologous hosts (bacteria, yeasts, mammalian and insect cells or transgenic animals and plants), allowing the comparison of the most efficient methods in terms of costs, product quality and safety.

Vaccinology is a rapidly expanding research field and new vaccination strategies have been developed thanks to modern technologies based on the rational design of attenuated pathogens, live recombinant vaccines and protein (antigen)- or peptide (epitope)-based subunit vaccines (Plotkin 2005). The aim of these approaches is to obtain efficient and safe vaccine formulations being able at the same time to induce effective, long lasting immunity against complex viral pathogens such as HIV-1 (Nabel 2001), influenza A/H5N1 virus (Stephenson et al. 2004), SARS-coronavirus (Stadler and Rappuoli 2005). To reach this goal, targets must be found able not only to generate long-term memory B cell producing neutralizing antibodies to block free pathogens, but also T cell mediated immunity for

P. Ranalli (ed.), Improvement of Crop Plants for Industrial End Uses, 417–433.

the control of pathogen spreading by the elimination of infected cells (Lambert et al. 2005).

The newborn branch of plant biotechnology defined as "molecular farming" is mainly focused on the exploitation of plants of agronomic relevance as factories for the large scale production of biomolecules. The possibility to produce subunit vaccines through plants paves new ways and offers solutions to some of the problems associated to traditional production systems.

2. Antigen production in plants: advantages and perspectives

2.1 Stable nuclear and plastid transformation

The stable expression of heterologous proteins in plants can be performed by inserting the exogenous encoding gene into nuclear or plastid genomes. To obtain plants transformed in the nuclear genome the natural capability of *Agrobacterium tumefaciens* to transfer DNA into plant cells (Tinland 1996) is amply exploited. By this method plant tissues can be transformed either in vitro, on small leaf explants, or directly in planta (Feldmann and Marks 1987; Bechtold and Pelletier 1998; Clough and Bent 1998). But the transformation of plant species less susceptible to this pathogen (i.e. some monocots), can be also achieved by shooting DNA-coated tungsten or gold microbeads on plant tissues through a "particle gun" (biolistic method) (Taylor and Fauquet 2002).

Several antigens have been successfully expressed in plants by stable nuclear transformation with different ends. In some cases, plants were considered only as "biofactories" for massive production, while in others edible plant varieties have been chosen for the direct oral delivery of the expressed antigens (Table 14.1).

Table 14.1. Representative antigens stably expressed in plants.

Pathogen	Antigen	Transgenic Plant	References
Hepatitis B Virus	Surface antigen	*Lactuca sativa* *Lupinus luteus* *Nicotiana tabacum* *Solanum tuberosum*	Mason et al. 1992; Thanavala et al. 1995; Ehsani et al. 1997; **Kapusta et al. 1999**; Richter et al. 2000; **Thanavala et al. 2005**

Table 14.1. Representative antigens stably expressed in plants.

Pathogen	Antigen	Transgenic Plant	References
Norwalk Virus	Capsid protein	*Nicotiana tabacum* *Solanum tuberosum*	Mason et al. 1996; **Tacket et al. 2000**
Enterotoxic E. coli	Heat-labile enterotoxin B subunit	*Nicotiana tabacum* *Solanum tuberosum* *Zea mays*	Haq et al. 1995; Mason et al. 1998 ; **Tacket et al. 1998**; **Tacket et al. 2004**
Rabies Virus	Glycoprotein	*Lycopersicon esculentum*	McGarvey et al. 1995
Cytomegalovirus	Glycoprotein B	*Nicotiana tabacum*	Tackaberry et al. 1999
Vibrio cholerae	Cholera toxin B subunit	*Solanum tuberosum*	Arakawa et al. 1997 ; Arakawa et al. 1998
Foot-and-mouth disease virus	VP1 Structural protein	*Arabidopsis thaliana*	Carrillo et al. 1998
Porcine transmissible gastroenteritis coronavirus	Glycoprotein S	*Arabidopsis thaliana* *Nicotiana tabacum* *Zea mays*	Gomez et al. 1998; Tuboly et al. 2000 ; Streatfield et al. 2001

In bold characters are indicated references describing clinical trials of the plant-expressed antigens.

The use of plants can indeed represent not only a measure to reduce production costs, improve product safety and eliminate the use of needles, but also a strategy to efficiently deliver vaccines at mucosal level (Tacket et al. 1998; Kapusta et al. 1999; Tacket et al. 2000; Tacket et al. 2004; Thanavala et al. 2005). Nonetheless, antigen production in plants still suffer some limitations of the nuclear transformation technology, basically due to the low and variable expression levels of the heterologous gene among independent lines. This is particularly detrimental when the direct oral delivery of the plant tissue is foreseen, as a precise a priori establishment of the vaccine dose is impracticable. A step taken to face this problem consists in optimizing the heterologous gene sequence by using host codon usage, removing cryptic genetic signals that can negatively affect post-translational steps and targeting the heterologous protein in appropriate subcellular compartments ensuring proper folding (Sullivan and Green 1993; Koziel et al. 1996). Another step consists in identifying suitable 5' and 3' regulatory elements able to ensure reproducible high-level and tissue-specific transgene expression, as it has been shown that the activity of nominally constitutive promoters can vary as a function of the developmental stage, organ and plant species (Williamson et al. 1989; Malik et al. 2002; Samac et al. 2004). Recently, new plant-derived regulatory sequences, such as the promoter-terminator

of the highly transcribed gene (*rbcS1*) of the ribulose-1,5-bisphosphate carboxylase (RBC) small-subunits gene family in chrysanthemum (Outchkourov et al. 2003), the regulatory sequences of the seed storage protein gene arcelin 5-I (arc5-I) of common bean (*Phaseolus vulgaris*) (De Jaeger et al. 2002) or novel gene regulatory elements associated with a cryptic constitutive promoter from tobacco (Malik et al. 2002), have been identified. These sequences are currently used to improve transgene expression levels in different plant species. Turned in a similar direction are those approaches aimed to the production of transgenic plant lines stably encoding replicating viral vectors, the so-called replicons or amplicons, and expressing uniformly and synchronously a foreign gene, thanks to the efficiency of viral replicases (Palmer et al. 1999; Mallory et al. 2002; Gleba et al. 2004; Zhang and Mason 2005).

Alternatively, efforts have been made to point out preferences in *Agrobacterium* T-DNA insertion target site and to evaluate whether transgene expression variability in single copy transgenic plants can be correlated with integration position (Gelvin 2000; De Buck et al. 2004; Schneeberger et al. 2005).

In a similar direction, efforts are oriented attempting to adjust gene targeting by homologous recombination in plants, to induce the integration of the heterologous gene into a pre-determined genomic location in a single copy pattern, preventing the accidental inactivation of genes fundamental for plant metabolism or the anomalous expression profiles due to post-transcriptional silencing (Hanin and Paszkowski 2003; Srivastava and Ow 2004). Progress in this sense has been achieved using Cre-*lox* mediated recombination mainly in tobacco and rice, in the latter case by optimizing the design of T-DNA flanking regions (Albert et al. 1995; Terada et al. 2002; Cotsaftis and Guiderdoni 2005).

An interesting alternative to nuclear stable transformation is the insertion of antigen encoding genes in the plastid genome (Tregoning et al. 2003; Koya et al. 2005; Molina et al. 2005; Glenz et al. 2006). Fundamental difference between plastid and nuclear transformation is that in the plastid homologous recombination of the foreign sequence is feasible, eliminating problems linked to “positional effect”. Moreover, the plastid genome present in several copies in a single cell, rapidly replicates ensuring high yields of recombinant protein production (Daniell et al. 2005). Despite these advantages, up to now no data are available regarding the oral delivery of transplastomic plant tissues, probably because fertile edible transplastomic plants are still under construction (Ruf et al. 2001; Lelivelt et al. 2005).

2.2 Transient transformation

To get round the difficulties encountered in obtaining high rate of expression of the heterologous gene in nuclear transformed plants, transient transformation strategies have been devised. In this case, the gene encoding the antigen of interest is inserted in the genome of a pathogen that is used as a vector for expression during plant infection. The two major techniques used to get transient transformation are based on plant viruses- or *Agrobacterium*-mediated infection of fully developed plants.

The use of plant viruses (typically ss(+)RNA viruses such as Tobacco Mosaic Virus (TMV), Cowpea Mosaic Virus (CPMV), Potato Virus X (PVX), and Alfalfa Mosaic Virus (AlMV)) to transiently express heterologous genes, has been greatly favored by the development of expression vectors harboring the cDNA of the complete viral genome (Pogue et al. 2000). Using these tools, the foreign gene can be easily inserted as an additional Open Reading Frame (ORF) or, when possible, by replacing unessential viral functions (Lacomme et al. 1998). By this way many antigens have been successfully produced able to induce in animal models good antibody responses, both when delivered in extracted and purified forms or directly per os using the infected plant tissues (Table 14.2).

Table 14.2. Representative antigens and epitopes transiently expressed in plants through viral vectors.

Pathogen	Antigen	Viral Vector	References
P. falciparum	Several epitopes	TMV	Turpen et al. 1995
Influenza virus	Hemagglutinin epitope	TMV	Sugiyama et al. 1995
HIV-1	Capsid epitopes	PVX CPMV	Porta et al. 1996 ; Yusibov et al. 1997 ; Marusic et al. 2001
S. aureus	Fibronectin binding protein epitope	PVX	Brennan et al. 1999
FMDV	VP1 Structural protein	TMV	Wigdorovitz et al.1999
Hepatitis B virus	Mimotope	TMV	Nemchinov et al. 2000
Rabies virus	Chimeric peptide	AlMV	**Yusibov et al. 2002**
HPV	E7 Oncoprotein	PVX	Franconi et al. 2002
BHV-1	Glycoprotein D	TMV	Pérez Filgueira et al. 2003
Colorectal cancer	GA733-2 Antigen	TMV	Verch et al. 2004
Hepatitis C virus	Mimotope	CMV	Natilla et al. 2004

Table 14.2. Representative antigens and epitopes transiently expressed in plants through viral vectors.

Pathogen	Antigen	Viral Vector	References
RSV	G protein	AlMV	Yusibov et al. 2005
HIV-1	Tat protein	TMV	Karasev et al. 2005
Y. pestis	F1 and V proteins	MagnICON	Santi et al. 2006

HIV-1: Human Immunodeficiency Virus type 1; *FMDV*: Foot and Mouth Disease Virus; *HPV*: Human Papilloma Virus; *BHV-1*: Bovine Herpes Virus type 1; *RSV*: Respiratoty Syncytial Virus; *TMV*: Tobacco Mosaic Virus; *PVX*: Potato Virus X; *CPMV*: Cowpea Mosaic Virus; *AlMV*: Alfalfa Mosaic Virus; *CMV*: Cucumber Mosaic Virus. In bold character is indicated the reference describing clinical trial of a plant-expressed chimeric peptide through a viral vector.

Up to now agroinfiltration technique has been used mainly to predict expression efficiency of constructs before their use for nuclear stable transformation. However, recent approaches converted this procedure into a valuable alternative expression system. For example, Icon Genetics Inc. (recently acquired by Bayer) has developed a system (magnICON) for the overexpression of foreign genes that conjugates the efficiency of *Agrobacterium*-mediated transformation with viral rate of expression (Marillonnet et al. 2004, 2005; Gleba et al. 2005; Gils et al. 2005). The strategy is based on the optimization of the TMV genome (by the removal of cryptic sequence that, if recognized by the plant machineries, could affect viral replication/spreading) and on the split of the improved genome into 5' and 3' modules of expression. The 5' module includes the viral polymerase and the movement proteins genes while the 3' module carries the gene of interest in substitution of the viral coat protein gene. These two modules, used to co-infiltrate plants, are linked together by a recombinase encoded by a third co-infiltrated vector that recognizes specific recombinase target-sequences, artificially added to each module. This "deconstructed" virus system that lacks the coat protein gene, is unable to spread throughout the plant and in the environment, is not influenced by the dimension of the inserted gene due to the absence of the packaging process, while leads the plant cell machinery to the production of the heterologous protein. Using this strategy high levels of different *Yersinia pestis* antigens have been expressed in *Nicotiana benthamiana* plants. These plant-produced antigens are efficient in inducing protective immune responses against the pathogen when administered subcutaneously to guinea pigs (Santi et al. 2006).

3. Peptide-based vaccine production in plants: new solutions to old problems

Epitopes are the most important parts of an antigen because, by interacting with antigen receptors of B and T cells, they are responsible for the induction of the immune response. The use of synthetic peptides corresponding in their sequence to linear epitopes has been considered for the development of safe vaccines. Unfortunately, the efficacy of peptides in the induction of an immune response is limited mainly because of their very short half-life in the serum. Several delivery systems have been designed to circumvent this limitation.

An attractive approach to produce epitope-based plant vaccines is the construction of chimeric plant viruses displaying on the surface of the assembled particles peptides of interest for vaccine formulations (Pogue et al. 2002). By this approach, plants are employed as biofactories and large scale "reservoirs" of chimeric virus particles (CVPs) carrying the epitope. To construct CVPs, the sequence coding for the heterologous peptide is fused to the viral coat protein (CP) gene in a position known to be exposed on viral surface (Johnson et al. 1997; Porta and Lomonossoff 1998). To guarantee the production of large quantities of CVPs, the structural and functional characterization of the CP is essential to place the foreign peptide avoiding the interference with viral assembly, stability and/or spreading through the plant. To this aim, fundamental are the studies that define the biochemical factors governing viral movement and CP/virus particle structures by mutational, immunological and X-ray diffraction/spectro-metry analysis (Carrington et al. 1996; Callaway et al. 2001; Bendahmane et al. 1999; Porta et al. 2003).

The efficacy of CVPs in inducing antibody responses specific to the displayed epitope have been extensively demonstrated. Purified CVPs administered to animal models intranasally, intraperitoneally, or orally by direct delivery of virus-infected plant tissues (in this case also in humans) have been able to induce strong specific neutralizing immune responses (Table 14.2).

Recent data indicate that CVPs could be able to induce also the activation of HLA class I restricted T cell responses and of Natural Killer cells (Yusibov et al. 2005). Endorsement of these findings envisages a significant expansion of plant derived CVPs in the field of vaccinology.

4. Efficient delivery of subunit (plant-derived) vaccines: will plants offer the solution?

Although subunit vaccines offer great perspectives, their development is somewhat impaired due to the fact that peptides and proteins are poorly immunogenic and/or unable to be properly presented to cytotoxic T cells (Sette and Fikes 2003). For this reason, aside from the identification of essential targets, most of the efforts are focused on the improvement of vaccine efficacy.

As far as the recombinant antigen-based formulations is concerned, a fundamental role is played by substances able to enhance the immune response against the antigen and fundamental to strengthen the efficacy of the active principle in the vaccine (adjuvants) (Cox and Coulter 1997). Research is currently in progress in this area. Nonetheless, aluminium salts, the first adjuvant described (Glenny et al. 1926), still remain the standard for human use.

Plants have also been considered as possible source of adjuvants. The most known plant-derived adjuvants are saponins and in particular the QS21 acylated 3,28-*o*-bisdesmodic triterpene saponin extracted from the bark of the South American tree *Quillaja saponaria* Molina (Jacobsen et al. 1996). Basically, the interest to this natural surfactant derives from its high water solubility and the stability (hours if not days) of the mixture with the antigen preparation. Delivered in combination with different antigens not only in several animal models, but also in human trials, QS-21 has been able to enhance both Th1and Th2 responses (Moore et al. 1999) and to favor the activation of cytotoxic T cells (Newman et al. 1997). Besides QS-21 and new variants of natural or semi-synthetic saponins (da Silva et al. 2005; Marciani et al. 2003), a broad range of different plant-derived compounds, such as the Neem tree Leaf Preparation (NLP) (Baral et al. 2005), a compound of Chinese herbal medicinal ingredients (cCHMIs) (Wang et al. 2005) and the Rb1 fraction of ginseng (Rivera et al. 2005), is under investigation to evaluate adjuvanticity. Some of these compounds together with molecules, such as triterpenoids (Squalene is at the moment the sole adjuvant, together with alum, approved for human use) (Singh and O'Hagan 1999; Stephenson et al. 2005) are as effective as Freund's complete or incomplete adjuvant, and able to induce balanced Th1 and Th2 immune responses and active immunity even towards tumor antigens (Baral et al. 2005).

Aside the identification of immune response potentiator, new routes of delivery, or alternative delivery strategies, are also considered as key factors to improve subunit vaccines efficacy. Mucosal immunizations, for example, represent an efficient alternative to traditional parenteral immunization, inducing not only secretory IgA responses, but also systemical antibody production and T-cell mediated immunity (Holmgren and Czerkinsky 2005). Moreover, this non-invasive, easy-delivered immunization procedure is performed without the use of needles thus preventing cross-contamination. Plant-derived antigens both stably and transiently expressed in edible tissues/organs have been delivered orally and the efficiency of this immunization procedure has been evaluated in terms of antibody production. In this context, chimeric plant virus particles offer interesting solutions to the problem of peptide-vaccine efficacy for two main reasons. They are functional carriers for peptide-delivery and have been demonstrated to be efficient immune response-inducers without the need of adjuvant both when delivered in purified form (Marusic et al. 2001) or when orally administered in infected plant tissues (Yusibov et al. 2002). Studies have been carried out in order to define the fate of CVPs in vivo, by investigating the distribution in mice of CPMV particles orally or intravenously delivered (Rae et al. 2005). The conclusion was that these icosahedric virions, that are the best characterized to be used as nanoparticles (Wang et al. 2002a, 2002b; Chatterji et al. 2004), are stable during transit in the gastrointestinal tract being able to disseminate systemically thereafter. CPMV particles were found in several tissues throughout the body, supporting the idea that they can be used also as orally bioavailable nanocapsule for the delivery of vaccines and more generally of therapeutics.

5. Conclusions

More than two centuries have passed from Edward Jenner first vaccination against smallpox. Since then, many advances and scientific conquests have been done in the field of vaccinology. However, despite this progress a lot of delicate issues still subsist. First of all, the biological safety of the product as the adverse effects of vaccine delivery are unfortunately very frequent. A second but not less important socio-economical issue is that an excessive cost of the available vaccines prevents the application of WHO guidelines tending to cancel the disparities in health rights still existing among industrialized and developing countries. The use of plants as “biofactories” could offer the solution to some of these problems.

Up to now, plants have been mainly considered attractive as alternative systems only for the production of subunit vaccines by giving emphasis to their advantages in terms of cost-reduction and intrinsic biological safety of the product. However, many indications suggest that efforts should be now concentrated to define how plants could be exploited in novel strategies for subunit and epitope vaccine delivery. This could not only enhance the efficiency of vaccination but, above all, improve global health equity favouring the diffusion of modern vaccines to the world‘s poorest countries.

The obvious question now is why this “friendly” and promising technology has difficulties to take off and capital investment in this sector is still so limited.

References

Albert H, Dale EC, Lee E, Ow DW (1995) Site-specific integration of DNA into wild-type and mutant *lox* sites placed in the plant genome. Plant J 7:649-659

Arakawa T, Chong DK, Merritt JL, Langridge WH (1997) Expression of cholera toxin B subunit oligomers in transgenic potato plants. Transgenic Res 6:403-413

Arakawa T, Chong DK, Langridge WH (1998) Efficacy of a food plant-based oral cholera toxin B subunit vaccine. Nat Biotechnol 16:292-297

Baral R, Mandal I, Chattopadhyay U (2005) Immunostimolatory neem leaf preparation acts as an adjuvant to enhance the efficacy of poorly immunogenic B16 melanoma surface antigen vaccine. Int Immunopharmacol 5:1343-1352

Bechtold N, Pelletier G (1998) In planta *Agrobacterium*-mediated transformation of adult *Arabidopsis thaliana* plants by vacuum infiltration. Methods Mol Biol 82: 256-266

Bendahmane M, Koo M, Karrer E, Beachy RN (1999) Display of epitopes on the surface of Tobacco Mosaic Virus: Impact of charge and isoelectric point of the epitope on virus-host interaction. J Mol Biol 290:9-20

Brennan FR, Gilleland LB, Staczek J, Bendig MM, Hamilton WD, Gilleland HE (1999) A chimeric plant virus vaccine protects mice against a bacterial infection. Microbiology 145:2061-2067

Callaway A, Giesman-Cookmeyer D, Gillock ET, Sit TL, Lommel SA (2001) The multifunctional capsid proteins of plant RNA viruses. Annu Rev Phytopathol 34:419-460

Carrington JC, Kasschau KD, Mahajan SK, Schaad MC (1996) Cell-to-cell and long-distance transport of viruses in plants. Plant Cell 8:1669-1681

Carrillo C, Wigdorovitz A, Oliveros JC, Zamorano PI, Sadir AM, Gomez N, Salinas J, Escribano JM, Borca MV (1998) Protective immune response to foot-and-mouth disease virus with VP1 expressed in transgenic plants. J Virol 72:1688-1690

Chatterji A, Ochoa WF, Paine M, Ratna BR, Johnson JE, Lin T (2004) New addresses on an addressable virus nanoblock: uniquely reactive Lys residues on Cowpea Mosaic Virus. Chem Biol 11:855-863

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J 16:735-743

Cotsaftis O, Guiderdoni E (2005) Enhancing gene targeting efficiency in higher plants: rice is on the move. Transgenic Res 14:1-14

Cox CJ, Coulter AR (1997) Adjuvants – a classification and review of their modes of action. Vaccine 15:248-256

Da Silva BP, Soares JBRC, Paraguai de Souza E, Palatnik M, Palatnik de Sousa CB, Parente JP (2005) Pulcherrima saponin, from the leaves of *Calliandra pulcherrima*, as adjuvant for immunization in the murine model of visceral leishmaniasis. Vaccine 23:1061-1071

Daniell H, Chebolu S, Kumar S, Singleton M, Falconer R (2005) Chloroplast-derived vaccine antigens and other therapeutic proteins. Vaccine 23:1779-1783

De Buck S, Windels P, De Loose M, Depicker A (2004) Single copy T-DNAs integrated at different positions in the *Arabidopsis* genome display uniform and comparable β-glucuronidase accumulation levels. Cell Mol Life Sci 61:2632-2645

De Jaeger G, Scheffer S, Jacobs A, Zambre M, Zobell O, Goossens A, Depicker A, Angenon G (2002) Boosting heterologous protein production in transgenic dicotyledonous seeds using Phaseolus vulgaris regulatory sequences. Nat Biotechnol 20:1265-1268

Ehsani P, Khabiri A, Domansky NN (1997) Polypeptides of hepatitis B surface antigen produced in transgenic potato. Gene 190:107-111

Feldmann K, Marks M (1987) *Agrobacterium*-mediated transformation of germinating seeds of *Arabidopsis thaliana*: a non-tissue culture approach. Mol Gen Genet 208:1-9

Franconi R, Di Bonito P, Dibello F, Accardi L, Muller A, Birilli A, Simeone P, Donà MG, Venuti A, Giorni C (2002) Plant-derived Human Papillomavirus 16 E7 oncoprotein induces immune response and specific tumor protection. Cancer Res 62:3654-3658

Gelvin SB (2000) *Agrobacterium* and plant genes involved in T-DNA transfer and integration. Annu Rev Plant Physiol Plant Mol Biol 51:223-256

Gils M, Kandzia R, Marillonnet S, Klimyuk V, Gleba Y (2005) High-yield production of authentic human growth hormone using a plant virus-based expression system. Plant Biotech J 3:613-620

Gleba Y, Marillonnet S, Klimyuk V (2004) Engeneering viral expression vectors for plants: the “full virus” and the “deconstructed virus” strategies. Curr Opin Plant Biol 7:182-188

Gleba Y, Klimyuk V, Marillonnet S (2005) Magnifection – a new platform for expressing recombinant vaccines in plants. Vaccine 23:2042-2048

Glenny AT, Pope CG, Waddington H, Wallace UJ (1926) The antigenic value of toxoid precipitated by potassium alum. J Pathol Bacteriol 29:31–40

Glenz K, Bouchon B, Stehle T, Wallich R, Simon MM, Warzecha H (2006) Production of a recombinant bacterial lipoprotein in higher chloroplasts. Nat Biotechnol 24:76-77

Gomez N, Carrillo C, Salinas J, Parra F, Borca MV, Escribano JM (1998) Expression of immunogenic glycoprotein S polypeptides from transmissible gastroenteritis coronavirus in transgenic plants. Virology 249:352-358

Gomord V, Faye L (2004) Posttranslational modification of therapeutic proteins in plants. Curr Opin Plant Biol 7:171-181

Hanin M, Paszkowski J (2003) Plant genome modification by homologous recombination. Curr Opin Plant Biol 6:157-162

Haq TA, Mason HS, Clements JD, Arntzen CJ (1995) Oral immunization with a recombinant bacterial antigen produced in transgenic plants. Science 268:714-716

Holmgren J, Czerkinsky C (2005) Mucosal immunity and vaccines. Nat Med 11:S45-S53

Jacobsen NE, Fairbrother WJ Kensil CR, Lim A, Wheeler DA,Powell MF (1996) Structure of the saponin adjuvant QS-21 and its base-catalyzed isomerization product by 1H and natural abundance 13C NMR spectroscopy. Carbohydr Res 280:1-14

Johnson J, Lin T, Lomonossoff G (1997) Presentation of heterologous peptides on plant viruses: genetics, structure and function. Annu Rev Phytopathol 35:67-86

Kapusta J, Modelska A, Figlerowicz M, Pniewski T, Letellier M, Lisowa O, Yusibov V, Koprowski H, Plucienniczak A, Legocki AB (1999) A plant-derived edible vaccine against hepatitis B virus. FASEB J 13:1796-1799

Karasev AV, Foulke S, Weelens C, Rich A, Shon KJ, Zwierzynski I, Hone D, Koprowski H, Reitz M (2005) Plant based HIV-1 vaccine candidate: Tat protein produced in spinach. Vaccine 23:1875-1880

Koya V, Moayeri M, Leppla SH, Daniell H (2005) Plant-based vaccine: mice immunized with chloroplast-derived anthrax protective antigen survive anthrax lethal toxin challenge. Infect Immun 73:8266-8274

Koziel MG, Carozzi NB, Desai N (1996) Optimizing expression of transgenes with an emphasis on plant-transcriptional events. Plant Mol Biol 32:393-405

Lacomme C, Smolenska S, Wilson TMA (1998) Genetic engineering and the expression of foreign peptides or proteins with plant virus-based vectors. In: Setlow JK (ed) Genetic Engineering: Principles and Methods. Plenum Press, New York, pp 225-237

Lambert P-H, Liu M, Siegrist C-A (2005) Can successful vaccines teach us how to induce efficient protective immune responses? Nat Med 11: S54-S62

Lelivelt CLC, McCabe MS, Newell CA, de Snoo CB, van Dun KMP, Birch-Machin I, Gray JC, Mills KHG, Nugent JM (2005) Stable plastid transformation in lettuce (*Lactuca sativa* L.) Plant Mol Biol 58:763-774

Malik K, Wu K, Li X-Q, Martin-Heller T, Hu M, Foster E, Tian L, Wang C, Ward K, Jordan M, Brown D, Gleddie S, Simmonds D, Zheng S, Simmonds J, Miki B (2002) A constitutive gene expression system derived from the tCUP cryptic promoter elements. Theor Appl Genet 105:505-514

Mallory AC, Parks G, Endres MW, Baulcombe D, Bowman LH, Pruss GJ, Vance VB (2002) The amplicon-plus system for high-level expression of transgenes in plants. Nat Biotechnol 20: 622-625

Marciani DJ, Reynolds RC, Pathak AK, Finley-Woodman K, May RD (2003) Fractionation, structural studies, and immunological characterization of the semi-synthetic *Quillaja* saponins derivative GPI-0100. Vaccine 21:3961-3971

Marillonnet S, Giritch A, Gils M, Kandzia R, Klimyuk V, Gleba Y (2004) In planta engineering of viral RNA replicons: efficient assembly by recombination of DNA modules delivered by *Agrobacterium*. Proc Natl Acad Sci USA 101: 6852-6857

Marillonnet S, Thoeringer C, Kandzia M, Klimyuk V, Gleba Y (2005) Systemic *Agrobacterium tumefaciens*-mediated transfection of viral replicons for efficient transient expression in plants. Nat Biotechnol 23:718-723

Marusic C, Rizza P, Lattanzi L, Mancini C, Spada M, Belardelli F, Benvenuto E, Capone I (2001) Chimeric plant virus particle as immunogens for inducing murine and human immune response against Human Immunodeficiency virus Type 1. J Virol 75:8434-8439

Mason HS, Lam DM, Arntzen CJ (1992) Expression of hepatitis B surface antigen in transgenic plants. Proc Natl Acad Sci USA 89:11745-11749

Mason HS, Ball JM, Shi JJ, Jiang X, Estes MK, Arntzen CJ (1996) Expression of Norwalk capsid protein in transgenic tobacco and potato and its oral immunogenicity in mice. Proc Natl Acad Sci USA 93:5335-5340

Mason HS, Haq TA, Clements JD, Arntzen CJ (1998) Edible vaccine protects mice against *Escherichia coli* heat-labile enterotoxin (LT): potatoes expressing a synthetic LT-B gene. Vaccine 16:1336-1343

McGarvey PB, Hammond J, Dienelt MM, Hooper DC, Fu ZF, Dietzschold B, Koprowski H, Michaels FH (1995) Expression of the rabies virus glycoprotein in transgenic tomatoes. Bio/Technology 13:1484-1487

Molina A, Veramendi J, Hérva-Stubbs S (2005) Induction of neutralizing antibodies by a tobacco chloroplast-derived vaccine based on a B cell epitope from canine parvovirus. Virology 342:266-275.

Moore A, McCarthy L, Mills KHG (1999) The adjuvant combination monophosphoryl lipid A and QS21 switches T cell responses induced with a soluble recombinant HIV protein from Th2 to Th1. Vaccine 17:2517-2527.

Nabel GJ (2001) Challenges and opportunities for development of an AIDS vaccine. Nature 410:1002-1007

Natilla A, Piazzolla G, Nuzzaci M, Saldarelli P, Tortorella C, Antonaci S, Piazzolla P (2004) Cucumber mosaic virus as carrier of a hepatitis C virus-derived epitope. Arch Virol 149:137-154

Nemchinov LG, Liang TJ, Rifaat MM, Mazyad HM, Hadidi A, Keith JM (2000) Development of a plant-derived subunit vaccine candidate against hepatitis C virus. Arch Virol 145:2557-2573

Newman MJ, Wu J-Y, Gardner BH, Anderson CA, Kensil CR, Recchia J, Coughlin RT, Powell MF (1997) Induction of cross-reactive cytotoxic T-lymphocyte responses specific for HIV-1 gp120 using saponin adjuvant (QS-21) supplemented subunit vaccine formulations. Vaccine 15:1001-1007

Outchkourov NS, Peters J, de Jong J, Rademarkers W, Jongsma MA (2003) The promoter-terminator of chrysanthemum *rbcS1* directs very high expression levels in plants. Planta 216:1003-1012

Palmer KE, Thomson JA, Rybicki EP (1999) Generation of maize cell lines containing autonomously replicating maize streak virus-based gene vectors. Arch Virol 144:1345-1360

Pérez Filgueira DM, Zamorano PI, Domìnguez MG, Taboga O, Del Médico Zajac MP, Puntel M, Romera SA, Morris TJ, Borca MV, Sadir AM (2003) Bovine herpes virus gD protein produced in plants using a recombinant tobacco mosaic virus (TMV) vector possesses authentic antigenicity. Vaccine 21:4201-4209

Plotkin SA (2005) Vaccines: past, present and future. Nat Med 11:S5-S11

Pogue GP, Lindbo JA, Garger SJ, Fitzmaurice WP (2002) Making an ally from an enemy: plant virology and the new agriculture. Annu Rev Phytopathol 40:45-74

Porta C, Spall VE, Lin T, Johnson JE, Lomonosoff GP (1996) The development of cowpea mosaic virus as a potential source of novel vaccines. Intervirology 39:79-84

Porta C, Lomonossoff GP (1998) Scope for using plant viruses to present epitopes from animal pathogens. Rev Med Virol 8:25-41

Porta C, Spall VE, Findlay KC, Gergerich RC, Farrance CE, Lomonossoff GP (2003) Cowpea mosaic virus-based chimaeras. Effects of inserted peptides on the phenotype, host range, and transmissibility of the modified viruses. Virology 310:50-63

Rae CS, Lhor IW, Wang Q, Destito G, Gonzalez MJ, Singh P, Thomas DM, Estrada MN, Powell E, Finn MG, Manchester M (2005) Systemic trafficking of plant virus nanoparticles in mice via the oral route. Virology 343:224-235

Richter LJ, Thanavala Y, Arntzen CJ, Mason HS (2000) Production of hepatitis B surface antigen in transgenic plants for oral immunization. Nat Biotechnol 18:1167-1171

Rivera E, Ekholm Pettersson F, Inganas M, Paulie S, Gronvik K-O (2005) The Rb1 fraction of ginseng elicits a balanced Th1 and Th2 immune response. Vaccine 23:5411-5419

Ruf S, Hermann M, Berger IJ, Carrer H, Bock R (2001) Stable genetic transformation of tomato plastids and expression of a foreign protein in fruit. Nat Biotechnol 19:870-875

Samac DA, Tesfaye M, Dornbusch M, Saruul P, Temple SJ (2004) A comparison of constitutive promoters for expression of transgenes in alfalfa (*Medicago sativa*). Transgenic Res 13:349-361

Santi L, Giritch A, Roy CJ, Marillonnet S, Klimyuk V, Gleba Y, Webb R, Arntzen CJ, Mason HS (2006) Protection conferred by recombinant *Yersinia pestis* antigens produced by a rapid and highly scalable plant expression system. Proc Natl Acad Sci USA 24:861-866

Schneeberger RG, Zhang K, Tatarinova T, Troukhan M, Kwok SF, Drais J, Klinger K, Orejudos, Macy K, Bhakta A, Burns J, Subramanian G, Donson J, Flavell R, Feldmann K (2005) *Agrobacterium* T-DNA integration in *Arabidopsis* is correlated with DNA sequence compositions that occur frequently in gene promoter regions. Funct Integr Genomics 5:240-253

Sette A, Fikes J (2003) Epitope-based vaccines: an update on epitope identification, vaccine design and delivery. Curr Opin Immunol 15:461-470

Singh M, O'Hagan D (1999) Advances in vaccine adjuvants. Nat Biotechnol 17:1075-1081

Srivastava V, Ow DW (2004) Marker-free site-specific gene integration in plants. Trends Biotechnol 22:627-629

Stadler K, Rappuoli R (2005) SARS: understanding the virus and development of rational therapy. Curr Mol Med 5:677-697

Stephenson I, Nicholson KG, Wood JM, Zambon MC, Katz JM (2004) Confronting the avian influenza threat: vaccine development for a potential pandemic. Lancet Infect Dis 4:499-509

Stephenson I, Bugarini R, Nicholson KJ, Podda A, Wood JM, Zambon MC, Katz JM (2005) Cross-reactivity to highly pathogenic avian influenza H5N1 viruses after vaccination with nonadjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: a potential priming strategy. J Infect Dis 191:1210-1215

Streatfield SJ, Jilka JM, Hood EE, Turner DD, Bailey MR, Mayor JM, Woodard SL, Beifuss KK, Horn ME, Delaney DE, Tizard IR, Howard JA (2001) Plant-based vaccines: unique advantages. Vaccine 19:2742-2748

Sugiyama Y, Hamamoto H, Takemoto S, Watanabe Y, Okada Y (1995) Systemic production of foreign peptides on the particle surface of tobacco mosaic virus. FEBS Lett 359:247-250

Sullivan ML, Green PJ (1993) Post-transcriptional regulation of nuclear-encoded genes in higher plants: the roles of mRNA stability and translation. Plant Mol Biol 23:1091-1104

Tackaberry ES, Dudani AK, Prior F, Tocchi M, Sardana R, Altosaar I, Ganz PR (1999) Development of biopharmaceuticals in plant expression systems: cloning, expression and immunological rectivity of human cytomegalovirus glycoprotein B (UL55) in seeds of transgenic tobacco. Vaccine 17:3020-3029

Tacket CO, Mason HS, Losonsky G, Clements JD, Levine MM, Arntzen JC (1998) Immunogenicity in humans of a recombinant bacterial antigen delivered in transgenic potato. Nat Med 4:607-609

Tacket CO, Mason HS, Losonsky G, Estes MK, Levine MM, Arntzen JC (2000) Human immune responses to a novel Norwalk virus vaccine delivered in transgenic potatoes. J Infect Dis 182:302-305

Tacket CO, Pasetti M, Edelman R, Howard JA, Streatfield S (2004) Immunogenicity of recombinant LT-B delivered orally to humans in transgenic corn. Vaccine 22:4385-4389

Taylor NJ, Fauquet CM (2002) Microparticle bombardment as a tool in plant science and agricultural biotechnology. DNA Cell Biol 21:963-977

Terada R, Urawa H, Inagaki Y, Tsugane K, Iida S (2002) Efficient gene targeting by homologous recombination in rice. Nat Biotechnol 20:1030-1034

Thanavala Y, Yang Y, Lyons P, Mason HS, Arntzen C (1995) Immunogenicity of transgenic plant-derived hepatitis B surface antigen. Proc Natl Acad Sci USA 92:3358-3361

Thanavala Y, Mahoney M, Pal S, Scott A, Richter L, Natarajan N, Goodwin P, Arntzen CJ, Mason HS (2005) Immunogenicity in humans of an edible vaccine for hepatitis B. Proc Natl Acad Sci USA 102:3378-3382

Tinland B (1996) The integration of T-DNA into plant genome. Trends Plant Sci 1:178-184

Tregoning JS, Nixon P, Kuroda H, Svab Z, Clare S, Bowe F, Fairweather N, Ytterberg J, Wijk KJ, Dougan G, Maliga P (2003) Expression of tetanus toxin Fragment C in tobacco chloroplasts. Nucleic Acid Res 31:1174-1179

Tuboly T, Yu W, Bailey A, Degrandis S, Du S, Erickson L, Nagy E (2000) Immunogenicity of porcine transmissible gastroenteritis virus spike protein expressed in plants. Vaccine 18:2023-2028

Turpen TH, Reinl SJ, Charoenvit Y, Hoffman SL, Fallarme V, Grill LK (1995) Malarial epitopes expressed on the surface of recombinant tobacco mosaic virus. Bio/Technology 13:53-57

Verch T, Hooper DC, Kiyatkin A, Steplewski Z, Koprowski H (2004) Immunization with a plant-produced colorectal cancer antigen. Cancer Immunol Immunother 53: 92-99

Wang D, Hu Y, Sun J, Kong X, Zhang B, Liu J (2005) Comparative study on adjuvanticity of compound Chinese herbal medicinal ingredients. Vaccine 23:3704-3708

Wang Q, Kaltgrad E, Lin T, Johnson JE, Finn MG (2002a) Natural supramolecular building blocks: wild-type Cowpea Mosaic Virus. Chem Biol 9:805-811

Wang Q, Lin T, Johnson JE, Finn MG (2002b) Natural supramolecular building blocks: cysteine-added mutants of Cowpea Mosaic Virus. Chem Biol 9:8135-819

Wigdorovitz A, Pérez Filgueira DM, Robertson N, Carrillo C, Sadir AM, Morris TJ, Borca MV (1999) Protection of mice against challenge with Foot and Mouth Disease Virus (FMDV) by immunization with foliar extracts from plants infected with recombinant tobacco mosaic virus expressing the FMDV structural protein VP1. Virology 264:85-91

Williamson JD, Hirsch-Wyncott ME, Larkins BA, Gelvin SB (1989) Differential accumulation of a transcript driven by the CAMV 35S promoter in transgenic tobacco. Plant Physiol 90:1570-1576.

Yusibov V, Modelska A, Steplewski K, Agadjanyan M, Weiner D, Hooper DC, Koprowsky H (1997) Antigens produced in plants by infection with chimeric plant viruses immunize against rabies virus and HIV-1. Proc Natl Acad Sci USA 94:5784-5788

Yusibov V, Hooper DC, Spitsin SV, Fleysh N, Kean RB, Mikheeva T, Deka D, Karasev A, Cox S, Randall J, Koprowski H (2002) Expression in plants and immunogenicity of plant virus-based experimental rabies vaccine. Vaccine 20:3155-3164

Yusibov V, Mett V, Mett V, Davidson C, Musiychuk K, Gilliam S, Farese A, MacVittie T, Mann D (2005) Peptide-based candidate vaccine against respiratory syncytial virus. Vaccine 23:2261-2265

Zang X, Mason H (2005) Bean Yellow Dwarf Virus replicons for high-level transgene expression in transgenic plants and cell cultures. Biotechnol Bioengineering 93:271-279

15

Molecular farming of antibodies in plants

Richard M. Twyman[1], Stefan Schillberg[2] and Rainer Fischer[2]

[1]Department of Biology, University of York, Heslington, York YO10 5DD, United Kingdom (e-mail: Richard@writescience.com) [2]Fraunhofer Institute for Molecular Biology and Applied Ecology (IME), Forckenbeckstrasse 6, 52074 Aachen, Germany (e-mail: rainer.fischer@ime.fraunhofer.de)

1. Introduction

Antibodies are multi-subunit glycoproteins, produced by the vertebrate immune system. They recognize and bind to their target antigens with great affinity and specificity, which allows them to be used for many applications, including the diagnosis, prevention and treatment of human and animal disease (Anderson and Krummen 2003; Chad and Chamow 2001; Fischer and Emans 2000). It is estimated that approximately 1000 therapeutic recombinant antibodies are under development, up to one quarter of which may already be undergoing clinical trials. A large proportion of these antibodies recognize cancer antigens but others have been developed for the diagnosis and treatment of infectious diseases, acquired disorders and even transplant rejection (Gavilondo and Larrick 2000). As well as biomedical applications, antibodies can also be exploited to prevent diseases in plants (Schillberg et al. 2001), to detect and remove environmental contaminants, and for various industrial processes such as affinity purification and molecular targeting (Stoger et al. 2005b).

With such a diverse spectrum of uses, the potential market for antibodies is extremely large and there is considerable interest in high-capacity production technologies that are robust, economical and safe. Over the last

P. Ranalli (ed.), Improvement of Crop Plants for Industrial End Uses, 435–469.

15 years, plants have emerged as a convenient, economical and scalable alternative to the mainstream antibody production systems which are based on the large-scale culture of microbes or animal cells (Chu and Robinson 2001; Wurm 2004). In this chapter, we discuss the advantages and disadvantages of plants for antibody production, the diverse plant-based systems which are now available, and factors governing the success of antibody production in plants. We begin, however, with a brief overview of recombinant antibody technology and the diversity of antibody formats and derivatives that can now be synthesized.

2. Recombinant antibody technology

2.1 Structure of natural antibodies

The typical antibody format is the mammalian serum antibody, which comprises two identical heavy chains and two identical light chains joined by disulfide bonds (Figure 15.1).

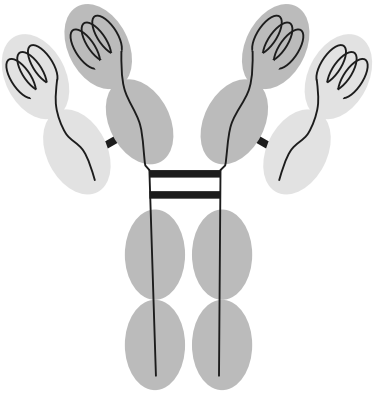


Fig. 15.1. Structure of a typical mammalian serum antibody, comprising two identical heavy chains (dark gray) and two identical light chains (light gray). Solid black lines indicate continuation of the polypeptide backbone (simple lines indicate the constant parts of the antibody, curly lines indicate the variable regions). Antibody domains are indicated by circles. Disulfide bonds are represented by thick black bars.

Each heavy chain is folded into four domains, two either side of a flexible hinge region which allows the multimeric protein to adopt its characteristic Y-shape. Each light chain is folded into two domains. The N-terminal domain

of each of the four chains is variable, i.e. it differs among individual B-cells due to unique rearrangements of the germ-line immunoglobulin genes. This part of the molecule is responsible for antigen recognition and binding. The remainder of the antibody comprises a series of constant domains, which are involved in effector functions such as immune cell recognition and complement fixation. Below the hinge, in what is known as the Fc portion of the antibody, the constant domains are class-specific. Mammals produce five classes of immunoglobulins (IgG, IgM, IgA, IgD and IgE) with different effector functions. The Fc region also contains a conserved asparagine residue at position 297 to which *N*-glycan chains are added. The glycan chains play an important role both in the folding of the protein and the performance of effector functions (Jefferis 2001).

Antibodies are also found in mucosal secretions, and these secretory antibodies have a more complex structure than serum antibodies. They are dimers of the serum-type antibody, the two monomers being attached by an additional component called the joining chain. There is also a further polypeptide called the secretory component, which protects the antibodies from proteases (Figure 15.2).

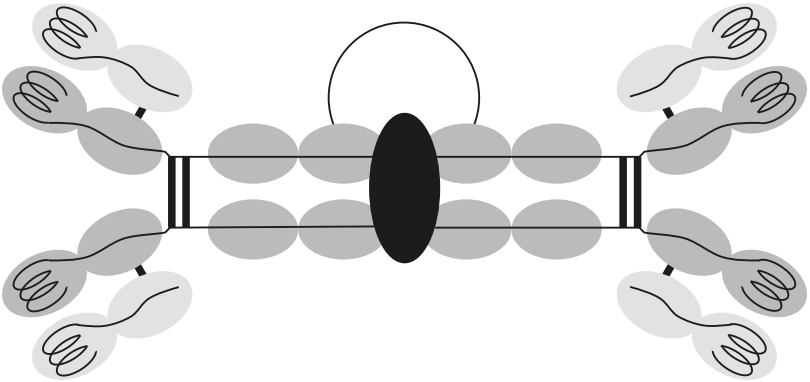


Fig. 15.2. Structure of a mammalian secretory antibody, comprising a dimer of the typical serum antibody, and including two additional components, the joining chain (black disc) and secretory component (white disc). Heavy chains are shown in dark gray and light chains in light gray. Solid black lines indicate continuation of the polypeptide backbone (simple lines indicate the constant parts of the antibody, curly lines indicate the variable regions). Antibody domains are indicated by circles. Disulfide bonds are represented by thick black bars.

2.2 Humanized recombinant monoclonal antibodies

Antibodies obtained from immunized animals are polyclonal, i.e. derived from many different B-cells. The advantage of monoclonal antibodies, i.e. antibodies derived from a single clone of B-cells, is that their binding specificity does not vary. The traditional source of monoclonal antibodies is murine B-cells. To provide a constant source of the antibody, B-cells of appropriate specificity are fused to immortal myeloma cells to produce a hybridoma cell line. However, the use of murine hybridoma-derived antibodies as therapeutics is limited because the murine components of the antibodies are immunogenic in humans, resulting in a so-called human anti-mouse antibody (HAMA) response. Therefore, numerous strategies have been developed to humanize murine monoclonal antibodies (Kipriyanov and Little 1999) culminating in the production of transgenic mice expressing the human immunoglobulin genes (Green 1999). An alternative approach is to use phage display libraries based on the human immune repertoires. Phage display is advantageous because high-affinity antibodies can be identified rapidly, novel combinations of heavy and light chains can be tested and the DNA sequence encoding the antibody is indirectly linked to the antibody itself (Griffiths and Duncan 1998; Sidhu 2000). This avoids the laborious isolation of cDNA or genomic immunoglobulin sequences from hybridoma cell lines.

2.3 Recombinant antibody derivatives

The expression of serum type or secretory type antibodies as recombinant molecules requires the preparation and expression of two and four different transgenes, respectively. However, this is often an unnecessary complication because in many cases the effector functions conferred by the constant regions are neither required nor desired. The constant regions of native immunoglobulins are not required for antigen binding, and the variable regions of the heavy and light chains can interact perfectly well when joined on the same polypeptide molecule (Chad and Chamow 2001; Fischer and Emans 2000). Smaller antibody derivatives which still require two chains include Fab and $F(ab')_2$ fragments (which contain only the sequences distal to the hinge region), and minibodies which contain only part of the constant portion of the molecule. Other derivatives such as large single chains, single chain Fv fragments (scFvs) and diabodies contain the variable regions of the heavy and light chains joined by a flexible peptide chain. Such derivatives are often more effective as drugs than full-length immunoglobulins because they show increased penetration of target tissues,

reduced immunogenicity and they are cleared from tissues more rapidly. Another variant is the camelid serum antibody, which is unique in that it only contains heavy chains. A full-size camelid antibody can therefore be expressed from a single transgene. Further, more specialized derivatives include bispecific scFvs, which contain the antigen-recognition elements of two different immunoglobulins and can bind to two different antigens, and scFv-fusions, which are linked to proteins with additional functions. Examples of all these antibody derivatives are shown in Figure 15.3.

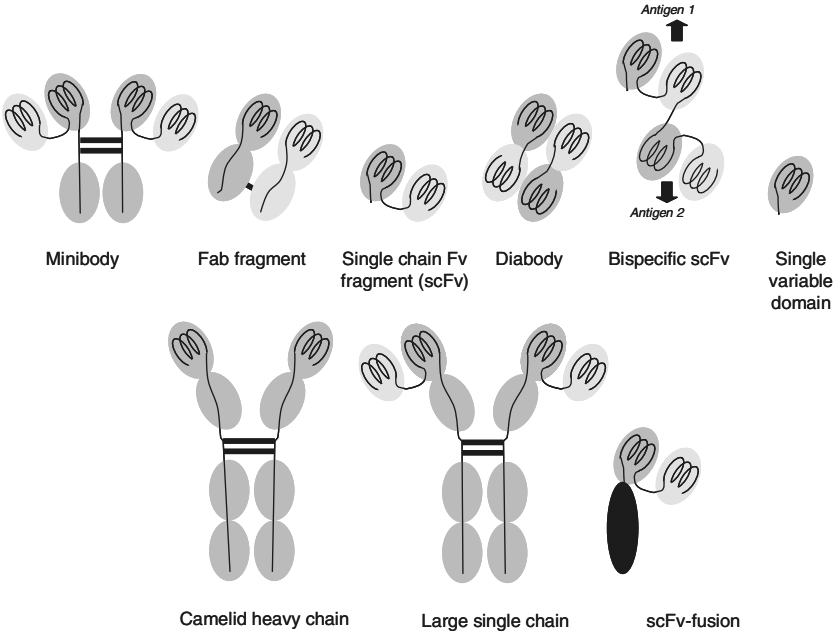


Fig. 15.3. Structure of recombinant antibody derivatives and atypical antibody formats, most of which have been expressed in plants. Heavy chain derivatives are shown in dark gray and light chain derivatives in light gray. Solid black lines indicate continuation of the polypeptide backbone (simple lines indicate the constant parts of the antibody, curly lines indicate the variable regions). Antibody domains are indicated by circles. Disulfide bonds are represented by thick black bars. The black disc indicates a new functional protein domain in the scFv fusion protein.

3. Expression systems for recombinant antibodies

3.1 Traditional expression systems

Most of the recombinant full-length immunoglobulins being developed as pharmaceuticals are produced in mammalian cell culture, a few in hybridoma lines but most in immortalized lines which have been cleared by the FDA and equivalent authorities in other countries. These lines include Chinese hamster ovary (CHO) cells, the murine myeloma cells lines NS0 and SP2/0, baby hamster kidney (BHK) and human embryonic kidney (HEK)-293 cells, and the human retinal line PER-C6 (Chu and Robinson 2001). The main reason for this is the belief that mammalian cells yield authentic products, particularly in terms of glycosylation patterns. However, there are minor differences in glycan chain structure between rodent and human cells. For example, human antibodies contain only the sialic acid residue N-acetylneuraminic acid (NANA) while rodents produce a mixture of NANA and N-glycosylneuraminic acid (NGNA) (Raju et al. 2000). There are many disadvantages to mammalian cell cultures, including the high set-up and running costs, the limited opportunities for scale-up and the potential contamination of purified recombinant antibodies with human pathogens. Bacterial fermentation systems are more cost effective than mammalian cell cultures and are therefore preferred for the production of Fab fragments and scFvs since these derivatives are not glycosylated. Even so, the yields of such products in bacteria are generally low because the proteins do not fold properly (Baneyx and Mujacic 2004). The main reason for sticking to these systems is that they are well characterized and established, and conform to the strict and extensive regulatory systems governing biopharmaceutical production.

Several alternative production systems have been explored, some of which are now well established while others are still experimental. In the former category, yeast and filamentous fungi have the advantages of bacteria (economy, robustness) but do have the tendency to hyper-glycosylate recombinant proteins (Gerngross 2004), while insect cells can be cultured in the same way as mammalian cells (although more cheaply) but also produce distinct glycan structures (Ikonomou et al. 2003). A more recent development is the production of antibodies in the milk of transgenic animals (Dyck et al. 2003). A disadvantage of animals, in common with cultured mammalian cells, is the existence of safety concerns about the transmission of pathogens or oncogenic DNA sequences. Finally, hen's eggs could also be used as a production system since they are protein rich and already synthesize endogenous antibodies, but they remain a relatively unexplored potential expression system (Harvey et al. 2002).

3.2 Antibody expression in plants

Plants offer a unique combination of advantages for the production of pharmaceutical antibodies (Twyman et al. 2003, 2005; Ma et al. 2003). The main benefit is the low production costs, reflecting the fact that traditional agricultural practices and unskilled labor are sufficient for maintaining and harvesting antibody-expressing crops. Also, large-scale processing infrastructure is already in place for most crops. Scale-up is rapid and efficient, requiring only the cultivation of more land. There are minimal risks of contamination with human pathogens.

The general eukaryotic protein synthesis pathway is conserved between plants and animals, so plants can efficiently fold and assemble full-size serum immunoglobulins (as first demonstrated by Hiatt et al. 1989) and secretory IgAs (first shown by Ma et al. 1995). In the latter case, four different subunits need to assemble in the same plant cell to produce a functional product, even though two different cell types are required in mammals. The post-translational modifications carried out by plants and animals are not identical to those in mammals, but they are very similar (certainly more so than fungal and insect systems). There are minor differences in the structure of complex glycans, such as the presence in plants of the residues α1,3-fucose and β1-2 xylose, which are absent from mammals (Cabanes-Macheteau et al. 1999). These residues are immunogenic in several mammals including humans, but curiously not in mice and only after multiple exposures in rats (Gomord et al. 2005; Faye et al. 2005). However, as discussed in more detail below, there are now many studies which show how the glycan profile of proteins produced in plants can be 'humanized'. As well as full-size antibodies, various functional antibody derivatives have also been produced successfully in plants, including Fab fragments, scFvs, bispecific scFvs, single domain antibodies and antibody fusion proteins (see Twyman et al. 2005).

4. Different plant-based expression systems

4.1 Nuclear transgenic plants (terrestrial crop species)

The most widely used strategy for antibody production in plants is the nuclear transgenic system, in which the antibody transgenes are transferred to the plant nuclear genome. The advantages of this approach when used in our major terrestrial crop species include: 1) transformation is a fairly routine procedure in many species and can be achieved by a range of methods,

the two most common of which are *Agrobacterium*-mediated transformation and the delivery of DNA-coated metal particles by microprojectile bombardment; 2) a stable transgenic line can be used as a permanent genetic resource; 3) among the various plant systems it is the simplest to maintain (once the producer line of transgenics is available) and is ultimately the most scalable; 4) it is possible to establish seed master banks. Disadvantages, compared to other plant systems, include the relatively long development time (required for transformation, regeneration, analysis of transgenics, selection and bulking up of the producer line), the unpredictable impact of epigenetic events on transgene expression (e.g. post-transcriptional gene silencing, position effects) and the potential for transgene spread from some crops through outcrossing. A range of different crops have been explored for antibody production, and the main categories are described below.

4.1.1 Leafy crop systems

Leafy crops have two major benefits – they have a large biomass which translates to large product yields, and flowering can be prevented (e.g. genetically, or by emasculation) to avoid the spread of transgenic pollen. On the other hand, leaf tissue is very watery, so proteins are expressed and accumulate in an aqueous environment in which they are subject to degradation. This means that antibody-containing leaves generally have to be processed soon after harvest, or otherwise frozen or dried which can add significantly to production costs. Tobacco (*Nicotiana tabacum*) has the longest history as a pharmaceutical production crop, having been used to express the very first plant-derived antibodies and many of the others since (Table 15.1).

The major advantages of tobacco are the well-established technology for gene transfer and expression, the high biomass yield (over 100,000 kg per hectare for close cropped tobacco, since it can be harvested up to nine times a year) and the existence of large-scale infrastructure for processing that does not come into contact with the human or animal food chains. Particularly due to the yield potential and safety features, tobacco could be a major source of plant-derived recombinant antibodies in the future.

Table 15.1. Recombinant therapeutic or diagnostic recombinant antibodies produced by molecular farming in plants and reported in the scientific literature (many antibodies in commercial development remain undisclosed until IP rights have been secured). Antibodies with alternative applications, such as phytomodulation or the prevention of plant disease, are not listed.

Antigen	Antibody format	Production system	Comments	References
B cell lymphoma, murine 38C13	scFv	Virus vectors in tobacco leaves	Maximum yield 30.2μg/g leaves	McCormick et al. (1999)
Carcinoembryonic antigen	scFv, IgG1	Tobacco agroinfiltration	Directed to apoplast or ER. Maximum yields 5μg scFv/g leaves, 1μg IgG/g leaves	Vaquero et al. (1999)
	dAb	Tobacco, agro-infiltration and transgenic plants		Vaquero et al. (2002)
	scFv	Rice, rice cell cultures	Directed to apoplast or ER. Maximum yields 3.8μg/g callus, 29μg/g leaves, 32μg/g seed	Torres et al. (1999), Stoger et al. (2000)
	scFv	wheat	Directed to apoplast or ER. Maximum yields900ng/g leaves, 1.5μg/g seed	Stoger et al. (2000)
	scFv	pea	Directed to rER. Maximum yield 9μg/g seed	Perrin et al. (2000)
CD-40	scFv-fusion	Tobacco suspension cells	Secreted to apoplast. Yield not reported.	Francisco et al. (1997)
Colon cancer antigen	IgG	Virus vectors in tobacco leaves	Yield not reported.	Verch et al. (1998)
Epidermal growth factor receptor (EGFR)	IgG	Tobacco	Aglycosylated antibody was directed to the ER and binds to EGFR expressed on the surface of human tumor cells.	Rodriguez et al. (2005)

Table 15.1. Continue:-

Antigen	Antibody format	Production system	Comments	References
Human creatine kinase	IgG1, Fab	Tobacco leaves Arabidopsis leaves	Accumulated in nucleolus or apoplast. Maximum yield 1.3% TSP.	De Neve et al. (1993), De Wilde et al. (1998)
	scFv	Tobacco leaves	Direct to cytosol or apoplast. Maximum yield 0.01% TSP	Bruyns et al. (1996)
Rhesus D antigen	IgG1	Arabidopsis leaves	Reacted with RhD^+ cells in antiglobulin technique and elicited a respiratory burst in human peripheral blood mononuclear cells.	Bouquin et al. (2002)
Ferritin	scFv	Tobacco leaves		Semenyuk et al. (2002)
Hepatitis B virus surface antigen	IgG	Tobacco leaves	Up to 25 mg antibody per kg biomass	Valdez et al. (2003a,b)
	IgG	Tobacco suspension cells	Complement-dependent cytotoxicity demonstrated	Yano et al. (2004)
Herpes simplex virus 2	IgG1	Soybean	Secreted to apoplast. Yield not reported.	Zeitlin et al. (1998)
HIV antibodies in blood	scFv-fusion	Tobacco leaves, barley grains, potato tubers	Maximum yield 150 μg/g	Schunmann et al. (2002)
Human choriogonadotrophin	scFv, dAb, IgG	Tobacco leaves	Secreted to apoplast. Maximum yield 40 mg/kg fresh weight	Kathuria et al. (2002)
	IgG1, diabody	Tobacco and winter cherry leaves	Directed to apoplast or ER. Glycan patterns were analyzed	Sriraman et al. (2004)
Human IgG	IgG1	Alfalfa	Secreted to apoplast. Maximum yield 1% TSP.	Khoudi et al. (1999)
Interleukin-4	scFv	Tobacco roots	Maximum yield 0.18% TSP	Ehsani et al. (2003)
Interleukin-6	scFv	Tobacco roots		Ehsani et al. (2003)

Table 15.1. Continue:-

Antigen	Antibody format	Production system	Comments	References
Protective antigen of Bacillus anthracis	IgG	N. benthamiana	Toxin activity was neutralized in vitro and in vivo.	Hull et al. (2005)
Rabies virus	IgG	Tobacco	Directed to the ER. Activity of the rabies virus was neutralized. Glycan patterns were analyzed.	Ko et al. (2003)
Salmonella enterica lipopolysaccharide	scFv	Tobacco	41.7 μg purified scFv per g leaf tissue	Makvandi-Nejad et al. (2005)
Streptococcal surface antigen (I/II)	sIgA	Tobacco leaves	Secreted to apoplast. Maximum yield 500 μg/g fresh weight	Ma et al. (1995)
	IgG1	Tobacco leaves	Directed to plasma membrane Maximum yield 1.1% TSP in leaves	Vine et al. (2001)
	IgG1	Secretion from tobacco roots	Up to 11.7 μg per gram dry root weight per day	Drake et al. (2003)
Substance P	VH	Tobacco leaves	Secreted to apoplast. Maximum yield 1% TSP.	Benvenuto et al. (1991)
Tetanus toxin C	IgG2a fused to tetanus toxin C	Tobacco	Animals immunized with recombinant immune complex without adjuvant were fully protected against lethal challenge	Chargelegue et al. (2005)
Tumor-associated antigen EpCAM	IgG	Tobacco	Secreted to the apoplast. Binding activity to colon cancer cells and tumor inhibition activity in nude mice.	Ko et al. (2005)

Another leafy crop that has been evaluated for antibody expression is alfalfa (*Medicago sativa*). This has been developed as a production crop by the Canadian biotechnology company Medicago Inc., and they have secured a robust IP portfolio covering the use of expression cassettes for biopharmaceutical proteins in this species. Although not as prolific as tobacco, alfalfa nevertheless produces large amounts of leaf biomass and has a high leaf protein content. Alfalfa also lacks the toxic metabolites produced in many tobacco cultivars which are often cited as a disadvantage, but instead it contains high levels of oxalic acid, which can affect protein stability. Alfalfa is particularly useful because it is a perennial plant that is easily propagated by stem cutting to yield clonal populations. Although alfalfa has been put on the biosafety 'hit list' by the regulators because it outcrosses with wild relatives, this does not detract from the excellent properties of this species for antibody production under containment. Alfalfa has been used for the production of a diagnostic IgG that recognizes epitopes specific to the constant regions of human IgG (Khoudi et al. 1999) and several further antibodies in development by Medicago Inc.

4.1.2 Dry seed crops

The problem of protein instability in leafy tissue (see above) can be overcome by expressing antibodies in the dry seeds of cereals and grain legumes. Several different species have been investigated for antibody production including four major cereals (maize, rice, wheat, barley) and two legumes (soybean, pea). The idea is that such crops would be beneficial for production in developing countries, where on-site processing would not be possible and a cold chain could not be maintained. The accumulation of recombinant antibodies in seeds allows long term storage at ambient temperatures because the proteins accumulate in a stable form. Seeds have the appropriate molecular environment to promote protein accumulation, and achieve this through the creation of specialized storage compartments such as protein bodies and storage vacuoles that are derived from the secretory pathway. Seeds are also desiccated, which reduces the level of both non-enzymatic hydrolysis and protease degradation. It has been demonstrated that antibodies expressed in seeds remain stable for at least three years at ambient temperatures with no detectable loss of activity (Stoger et al. 2005a).

As well as their advantages in terms of product stability, seed expression might also be beneficial in terms of downstream processing because seeds have a relatively simple proteome (therefore minimizing the likelihood that endogenous proteins would be co-purified) and lack the phenolic compounds abundant in leaves that can interfere with affinity purification. The restriction of recombinant protein accumulation to seeds also helps to

avoid any potentially negative effects on the growth and development of vegetative plant organs, and on animals and micro-organisms that interact with the plant or feed on its leaves.

Disadvantages of seed crops include the lower overall yields that have been obtained. The intrinsic yields are in a few cases higher than tobacco (e.g. on a kilogram per kilogram basis of harvested material, rice grains can accumulate more antibody than tobacco leaves (Stoger et al. 2002), but the vast abundance of harvested biomass per hectare from a tobacco crop far outweighs this. Also, seeds are regarded as viable genetically modified organisms in their own right, so while the transport of harvested transgenic tobacco leaves should not cause any problems, the transport of seeds could fall foul of national and international regulations on the transport of GMOs; the seeds would have to be crushed to flour beforehand and this might offset the advantage of increased product longevity.

Maize seeds have been investigated as an antibody production vehicle by Prodigene Inc., following successful demonstrations of the economical production of other valuable proteins using this system, including avidin and β-glucuronidase. Initial findings for the expression of a secretory IgA in maize showed that the four chains were expressed, directed to the cell wall matrix and assembled correctly. The product accumulated to 0.3% total soluble protein in the T1 seeds, and based on previous results, significant improvements were anticipated though selective breeding (Hood et al. 2002). An antibody derivative used for HIV diagnostics has been expressed in barley, and has achieved a yield of 150 $\mu g\ g^{-1}$.

Finally, antibodies have also been produced in soybean, although in this particular case it was expressed constitutively and isolated from the leaves rather than from the seeds (Zeitlin et al. 1998). Soybean has been investigated as a potential production crop by Prodigene and others because it is a self-fertilizing crop with a high biomass yield, but product yields have been low and the system has been largely abandoned.

4.1.3 Fruit and vegetable crops

Recombinant antibodies have been produced in potato and tomato, which also offer certain advantages over other crops. Proteins accumulating in potato tubers are generally stable, because like the cereal seed endosperm, these are storage organs that are adapted for high level protein accumulation. The potential of potato tubers for antibody production was first demonstrated by Artsaenko et al. (1998), who produced a scFv fragment specific for the inflammatory agent oxazolone. Potatoes have since been developed as a general production host for antibodies (De Wilde et al. 2002) as well as other biopharmaceuticals based on antibodies (Schunmann et al. 2002). Fruit crops have another potential advantage, which is

antibody expression in organs that are consumed raw allows the direct oral administration of recombinant antibodies designed for passive immunotherapy, such as protection of the oral cavity against pathogens. Stoger et al. (2002) describe preliminary experiments in which scFv84.66, recognizing the carinoembryonic antigen (CEA), is expressed in tomato fruits, although the accumulation levels were rather low (0.3 μg per gram fresh weight). Other advantages of tomato include the high biomass yields (about 68,000 kg per hectare, approaching the yields possible in tobacco) and the increased containment offered by growth in greenhouses.

4.2 The chloroplast transgenic system (terrestrial crop species)

Instead of introducing transgenes into the nuclear genome, they can be targeted to the chloroplast genome using particle bombardment or another physical DNA delivery technique and ensuring the transgene is embedded in a chloroplast DNA homology region (Maliga 2003, 2004). The main benefits of the chloroplast system are that there are thousands of chloroplasts in a typical leaf cell, yet only one nucleus – therefore, the number of transgene copies in the cell following plastid transformation and the establishment of homoplasmy is much higher promising greater product yields. This is enhanced by the absence of epigenetic phenomena such as transgene silencing in the chloroplast genome. Chloroplasts, derived from ancient bacteria, also support operon-based transgenes allowing the expression of multiple proteins from a single transcript. Finally, and perhaps most importantly from the regulatory perspective, chloroplasts are absent from the pollen of most of our food crops, which limits the potential for outcrossing (Daniell et al. 2005a).

There are two disadvantages to the chloroplast system – first, chloroplast transformation is not a standard procedure and is thus far limited to a relatively small number of crops (tobacco, tomato, potato, cotton, soybean and most recently lettuce and cauliflower (Daniell et al. 2005b; Lelivelt et al. 2005; Nugent et al. 2006). Secondly, since chloroplasts are derived from ancient bacteria they lack much of the eukaryote machinery for post-translational modification, i.e. they are unable to synthesize glycan chains. For this reason, they would be suitable for the production of scFvs but not full-size immunoglobulins.

In the only published report thus far dealing with antibody expression in terrestrial plant chloroplasts, a camelid antibody fragment was expressed in tobacco using an inducible T7-promoter system. Transcripts could be detected but no protein (Magee et al. 2004). However, antibodies have been expressed successfully in algal chloroplasts (see below).

4.3 Antibody production by transient expression (leafy crops)

Transient expression assays are generally used to evaluate the activity of expression constructs or test the functionality of a recombinant protein before committing to the long term goal of generating transgenic plants. However, transient expression can also be used as a routine production method if enough protein can be produced to make the system economically viable. The advantages of this approach include the minimal set-up costs and the rapid onset of protein expression, but scaling up is expensive and impractical so this type of system is particularly useful for the production of high-value proteins such as therapeutic antibodies, which have specialized markets and are required in small amounts.

An example of a transient expression system is the agroinfiltration method, where recombinant *Agrobacterium tumefaciens* are infiltrated into tobacco leaf tissue under vacuum and milligram amounts of protein can be produced within a few weeks (Kapila et al. 1997). This system has also been developed in alfalfa by Medicago researchers (D'Aoust et al. 2004) and is applicable in many other leafy species (Markus Sack, personal communication). Although stable transformation occurs at very low efficiency, many cells are initially transiently transformed only for the exogenous DNA to get diluted and degraded. However, before this happens, most cells contain the T-DNA and can express any transgenes carried therein. As extrachromosomal constructs, these unintegrated T-DNAs are free from position effects and epigenetic silencing phenomena that often reduce of abolish the expression of integrated nuclear transgenes.

A number of different antibodies and their derivatives have been produced by agroinfiltration, including the full-size IgG T84.66 along with its scFv and diabody derivatives (Vaquero et al. 1999, 2002) and a chimeric full-size IgG known as PIPP, along with its scFv and diabody derivatives, which recognizes human chorionic gonadotropin (Kathuria et al. 2002).

4.4 Plant viruses as expression vectors

Plant viruses are advantageous for the production of antibodies because viral genomes are easier to manipulate than plant genomes, and the infection of plants with recombinant viruses is a very simple process compared to the regeneration of transgenic plants. Potentially, plants carrying recombinant viruses can be grown on the same scale as transgenic plants, but with a much shorter development time. Viral infections are generally systemic, so infected plants carry the virus in all cells and can produce the antibody systemically, resulting in potentially very high yields. A further advantage of viruses is that mixed infections are possible, making it a simple process

to express, for example, the multiple chains of a full-size immunoglobulin. Although the transgene is carried on a viral genome rather than in the plant genome, the expressed protein is processed in the same manner as it would be in transgenic plants, meaning that appropriate folding, targeting and modification of antibodies is possible. The viral system is therefore uniquely simple, flexible and efficient, and has the potential for protein manufacture in both contained and open facilities (Canizares et al. 2005; Yusibov et al. 2006).

There are two types of expression systems based on plant viruses, one for full polypeptides and one for peptide epitopes displayed on the virion surface. Both have been used to express antibodies. In the polypeptide expression system, the antibody is encoded by a discrete transgene, and accumulates as a soluble protein within the plant cell. In the epitope display system, a small antibody derivative such as a scFv is expressed as a fusion with the viral coat protein in such a way that the antibody is displayed on the surface of the virus particle.

Tobacco mosaic virus (TMV) has a monopartite RNA genome of 6.5 kb encoding four proteins all of which are essential for systemic infection. The normal strategy for polypeptide expression is to place the transgene under the control of an additional coat protein promoter, although not a perfect copy of the endogenous coat protein promoter as this is an unstable configuration that leads to transgene elimination (Donson et al. 1991). Many antibodies have now been expressed in TMV-infected plants. McCormick et al. (1999) produced a scFv fragment based on the idiotype of malignant B-cells of the murine 38C13 B-lymphoma cell line. When administered to mice, the scFv stimulated the production of anti-idiotype antibodies capable of recognizing 38C13 cells, providing immunity against lethal challenge with the lymphoma. This has been developed into a personalized therapy for diseases such as non-Hodgkin's lymphoma, where antibodies capable of recognizing unique markers on the surface of any malignant B-cells could be produced for each patient. Up to 15 such antibodies were tested in phase I and phase II clinical trials by the US biotechnology company Large Scale Biology Inc. before they went into liquidation. Additionally, Verch et al. (1998) produced a full length IgG in transgenic tobacco plants by infecting them with two TMV vectors, one expressing the heavy chain and one the light chain. This study showed that viral coexpression was compatible with the correct assembly and processing of multimeric recombinant proteins.

Potato virus X (PVX), the type member of the *Potexvirus* family, has a 6.5-kb monopartite RNA genome rather like that of TMV. Also like TMV, PVX vectors contain extra subgenomic promoters to drive transgene expression but in this case the lack of a closely-related alternative means that transgene elimination by homologous recombination is unavoidable. PVX

vectors have been used for the expression of several different antibodies, but none of medical relevance. Single chain Fv antibodies have been expressed, specific for proteins from potato virus V (Hendy et al. 1999), tomato spotted wilt virus (Franconi et al. 1999) and against granule-bound starch synthase I (Ziegler et al. 2000).

In addition to the use of complete viruses carrying additional foreign genes, another strategy uses deconstructed viruses that cannot spread systemically in the plant. The magnifection strategy, developed by Icon Genetics (now part of Bayer CropSciences), renders the systemic spread of the virus unnecessary through the use of *A. tumefaciens* as a delivery vehicle (Marillonnet et al. 2005; Gleba et al. 2005). The bacterium delivers the viral genome to so many cells that local spreading is sufficient for the entire plant to be infected. Like the infection stage, systemic spread is a limiting function, often one of the primary determinants of host range. Taking the systemic spreading function away from the virus and relying instead on the bacterium to deliver the viral genome to a large number of cells allows the same viral vector to be used in a wide range of plants. The system has been used to express antigens and antibodies at high levels in tobacco and other plants (Gleba et al. 2004).

4.5 Systems based on the culture of plants, plant cells and plant tissues

The above systems all involve the use of whole plants as the expression platform. Even if antibody production is limited to specific tissues, such as seeds or leaves, these are harvested from the whole plant at the beginning of downstream processing. An alternative is to culture the specific organs or cells that produce the antibody, and either isolate the antibody from these cells or tissues, or collect it from the culture medium. A number of different culture systems have been developed, although most research has focused on cell suspension cultures.

We start with an example of 'whole plant culture'. In most cases where nuclear transgenic plants have been used for the production of recombinant antibodies, the product has been extracted from plant tissues. An alternative is to attach a signal peptide to the recombinant protein thus directing it to the secretory pathway. In this way, the protein can be recovered from the root exudates or leaf guttation fluid, processes known respectively as rhizosecretion and phyllosecretion (Borisjuk et al. 1999; Komarnytsky et al. 2000). Although not widely used, the secretion of recombinant antiobodies into hydroponic culture medium is advantageous because no cropping or harvesting is necessary. The technology is being developed by the US biotechnology company Phytomedics Inc. A monoclonal antibody

was shown to be secreted into hydroponic culture medium resulting in a yield of 11.7 μg antibody per gram of dry root mass per day (Drake et al. 2003).

Other systems are based on the culture of plant organs. Hairy roots are neoplastic structures that arise following transformation of a suitable plant host with *Agrobacterium rhizogenes*. If the plant is already transgenic, or if the transforming *A. rhizogenes* strain is transgenic and transfers the foreign gene to the host plant during the process of transformation, then hairy root cultures can be initiated which will produce recombinant antibodies and secrete them into the growth medium (Sharp and Doran 2001b). Hairy roots grow rapidly and can be propagated indefinitely in liquid medium. Thus far, hairy root cultures have been used to produce a relatively small number of antibodies (Sharp and Doran 2001a) mainly because of the relative ease with which multi-subunit proteins can be produced. The cultures can be initiated from transgenic plants already carrying multiple transgenes, wild type plants can be infected with multiple *A. rhizogenes* strains, or established hairy root cultures can be super-transformed with *A. tumefaciens*. A clonal root system based on a similar principle has been developed as a commercial platform by the Fraunhofer Center for Molecular Biotechnology in Newark, Delaware. In this case, the root system is combined with the use of viral-derived vectors for high-yield antibody expression in sealed vessels.

A tissue culture system has been developed from shooty teratomas, which are differentiated cell cultures produced by transformation with certain strains of *A. tumefaciens* (Subroto et al. 1996). Thus far, there has been only one report of pharmaceutical protein production in teratoma cultures, and the levels of antibody were very low (Sharp & Doran 2001a,b).

As stated above, most of the work in this area has focused on suspension cells, which are individual plant cells and small aggregates thereof growing in liquid medium in a fermenter (Hellwig et al. 2004; Doran 2006). Suspension cell cultures are usually derived from callus tissue by the disaggregation of friable callus pieces in shake bottles, and are later scaled up for fermenter-based production. Recombinant antibody production is achieved by using transgenic explants to derive the cultures, or transforming the cells after disaggregation, usually by co-cultivation with *A. tumefaciens*. Suspension cultures have the same advantages as the simple plants i.e. controlled growth conditions, batch-to-batch reproducibility, containment and production under GMP procedures. Many foreign proteins have been expressed successfully in suspension cells, including antibodies, enzymes, cytokines and hormones (reviewed by Hellwig et al. 2004, Fischer et al. 1999). Tobacco cultivar Bright Yellow 2 (BY-2) is the most popular source of suspension cells for molecular farming, since these proliferate

rapidly and are easy to transform. However, rice suspension cells have also been used to produce several antibodies (e.g. Torres et al. 1999).

Recombinant antibodies expressed in plant cell suspension cultures may be secreted into the culture supernatant or retained within the cells. Localization depends on expression construct design (see below) and the permeability of the plant cell wall to the antibody. Targeting signals included in the expression construct can be used to direct the protein to the apoplast or to retain it within intracellular compartments. The fate of antibodies targeted for secretion depends to a large extent on their size: molecules of 20–30 kDa (the size range of scFvs) will generally pass through the plant cell wall and be secreted into the culture medium, whereas larger proteins (such as IgGs) will be retarded in a size-proportional manner. The inclusion of a C-terminal KDEL sequence results in higher levels of antibody accumulation in cultured cells because the biochemical environment of the endoplasmic reticulum favors stable protein folding and assembly while reducing the level of proteolytic degradation (see below). However, this also makes it necessary to disrupt the cells in order to isolate the protein, which requires additional processing time and causes the release of phenolic molecules that interfere with purification and reduce production yield. Thus, the preferred approach is to secrete the target proteins and capture them from the culture supernatant or release them from the cells by mild enzymatic digestion.

4.6 Microbial and aquatic plants

Single-celled plants and aquatic plants can be maintained in bioreactors, offering two advantages over terrestrial plants. First, the growth conditions can be controlled precisely, which means that optimal growth conditions can be maintained, batch-to-batch product consistency improved and the growth cycle can conform to GMP. Second, growth in bioreactors offers complete containment. Although more expensive than agricultural molecular farming, the use of simple plants in bioreactors is not as expensive as cultured animal cells because the media requirements are generally very simple. Added to this, the proteins can be secreted into the medium, which reduces the downstream processing costs and allows the product to be collected in a non-destructive manner. A final, major advantage is the speed of production. The time from transformation to first product recovery is on the scale of days to weeks because no regeneration is required, and stable producer lines can be established in weeks rather than months to years because there is no need for crossing, seed-collection and the testing of several filial generations to check transgene stability. Three major bioreactor-

based systems are currently under commercial development: algae, moss and duckweed.

Thus far, a single report discusses the production of monoclonal antibodies in the chloroplast of the alga *Chlamydomonas reinhardtii* (Mayfield et al. 2003). Production costs appear similar to those of recombinant proteins produced in terrestrial plants, mainly due to the inexpensive media requirements (the medium does not cost very much to start with, and in any case can be recycled for algal cultures grown in continuous cycles). Aside from the economy of producing recombinant proteins in algae, there are further attributes that make algae ideal candidates for recombinant protein production. First, transgenic algae can be generated quickly, requiring only a few weeks between the generation of initial transformants and their scale up to production volumes. Second, both the chloroplast and nuclear genome of algae can be genetically transformed, providing scope for the production of several different proteins simultaneously. In addition, algae have the ability to be grown on various scales, ranging from a few milliliters to 500,000 liters in a cost-effective manner. These attributes, and the fact that green algae fall into the GRAS (generally regarded as safe) category, make *C. reinhardtii* a particularly attractive alternative to other plants for the expression of recombinant proteins. The production technology has been reviewed recently (Franklin and Mayfield 2005; Mayfield and Franklin 2005).

The moss *Physcomitrella patens* is a haploid bryophyte which can be grown in bioreactors in the same way as algae, suspension cells and aquatic plants. Like these other systems, it has the advantages of controlled growth conditions, synthetic growth media, and the ability to secrete recombinant proteins into the medium (Decker and Reski 2004). The unique feature of this organism, relative to all other plants, is that it is amenable to homologous recombination (Schaefer 2002). This means that not only can it be transformed stably with new genetic information, but that endogenous genes can be disrupted by gene targeting. The major application of gene targeting in molecular farming is the modification of the glycosylation pathway (by knocking out enzymes that add non-human glycan chains to proteins) thus allowing the production of humanized glycoproteins (Faye et al. 2005).

The *P. patens* system is being developed by the German biotechnology company Greenovation Biotech GmbH, which is based in Freiburg. The company has developed transient expression systems that allow feasibility studies, and stable production strains that can be scaled up to several thousand liters.

The Lemna System is based on duckweed (*Lemna minor*) and has been developed by the US biotechnology company Biolex Inc. Lemna has a number of significant advantages for the production of recombinant

pharmaceutical proteins (Gasdaska et al. 2003). Unlike transgenic terrestrial plants, this aquatic plant is cultured in sealed, aseptic vessels under constant growth conditions (temperature, pH and artificial light). Only very simple nutrients are required (water, air and completely synthetic inorganic salts) and under these conditions, the plant proliferates vegetatively and doubles its biomass every 36 hours. This provides the optimal production environment for batch-to-batch consistency. Duckweed constitutes about 30% dry weight of protein, and recombinant proteins can either be extracted from wet plant biomass or secreted into the growth medium. Biolex Inc. has reported the successful expression of tens of proteins in this system, including several recombinant antibodies and enzymes (Gasdaska et al. 2003).

5. Optimizing antibody production in plants

5.1 Design of the expression construct

The intrinsic production capacity of the chosen expression platform is a property that cannot be modified easily, because it is dependent on the overall biomass yield of the crop. However, the specific yield of recombinant protein per unit of plant biomass can be influenced by the optimization of transgene expression, which is achieved through expression construct design. Perhaps the most important component of the expression construct is the promoter used to control transcription of the transgene. For dicotyledonous species such as tobacco, potato and tomato, the strong and constitutive cauliflower mosaic virus 35S promoter (CaMV 35S) is often chosen to drive transgene expression (Twyman et al. 2005). In cereals, the CaMV 35S promoter has a lower activity and other promoters have been tested such as the maize ubiquitin-1 (ubi-1) promoter (Christensen and Quail 1996). Some modified dicot promoters do work rather well in cereals, an example being the pPLEX series of constructs developed by Schunman et al. (2003) adapted for use in monocots. The original pPLEX vectors were based on regulatory elements from subterranean clover stunt virus (SCSV). Modification was achieved by adding either the *Ubi1* or *Act1* introns, as well as GC-rich enhancer sequences from banana bunchy top virus (BBTV) or maize streak virus (MSV).

Regulated promoters can be used in preference to constitutive promoters to improve practicality and biosafety in addition to yields. For example, although constitutive promoters allow high-level accumulation of recombinant proteins in seeds, the proteins are also expressed in leaves, pollen and roots. The use of seed-specific promoters largely restricts recombinant

protein accumulation to the seeds, so the vegetative organs do not accumulate detectable levels of the recombinant protein. This increases the biosafety of the plants, since adventitious contact with non target organisms is unlikely (Commandeur et al. 2003). Among the many different seed-specific promoters that have been used (reviewed by Christou et al. 2004) the most impressive yields have been obtained with a novel seed-specific promoter from the common bean (*Phaseolus vulgaris*) which was used to express a single chain antibody in *Arabidopsis thaliana*. In contrast to the CaMV 35S promoter, which resulted in antibody accumulation to 1% total soluble protein (TSP), the bean *arc5-I* promoter resulted in antibody levels in excess of 36% TSP in homozygous seeds, and the antibody retained its antigen binding activity and affinity (De Jaeger et al. 2002).

The use of inducible promoters (Padidam 2003) is also advantageous because recombinant protein synthesis can be delayed until just before harvest, or even after harvest as is the case for the tomato hydroxy-3-methylglutaryl CoA reductase 2 (HMGR2) promoter developed by the now defunct CropTech Inc. as the MeGA promoter system (mechanical gene activation). The promoter used in this system is wound-inducible, and gene expression is activated when the harvested tobacco leaves are shredded prior to protein extraction (Cramer et al. 1999). However, many endogenous inducible promoters show a degree of leakiness (background expression) and in some cases a low induction ratio. Recombinant systems such as those based on bacterial operons or animal hormones may be advantageous in these circumstances.

After promoter choice, the next most important aspect of construct design is the inclusion of sequences that control subcellular targeting of the protein. This is a general method to increase the yield of recombinant proteins because the compartment in which a recombinant protein accumulates influences its folding, assembly and post-translational modification (Ma et al. 2003; Schillberg et al. 2003). Comparative targeting experiments with full-size immunoglobulins and single chain Fv fragments have shown that the secretory pathway is a more suitable compartment for folding and assembly than the cytosol, and is therefore advantageous for high-level protein accumulation (Zimmermann et al. 1998; Schillberg et al. 1999). The endoplasmic reticulum (ER) provides an oxidizing environment and an abundance of molecular chaperones, while there are few proteases. Proteins are directed to the secretory pathway using either a heterologous or endogenous signal peptide, located at the N-terminus of the native protein. Such proteins are cotranslationally imported into the ER and are eventually secreted to the apoplast, a supracellular network of interlinked compartments underlying the cell wall. Depending on its size, a protein can be retained in the cell wall matrix or it can leach from the cell. Although the

majority of recombinant proteins are generally more stable in the apoplast than the cytosol, they are even more stable in the ER lumen. Therefore, antibody expression levels can be increased even further if the protein is retrieved to the ER using an H/KDEL C-terminal tetrapeptide tag in addition to the signal peptide (Conrad and Fiedler 1998). Accumulation levels are generally two to tenfold greater compared with an identical protein lacking the KDEL signal (Schillberg et al. 2002). As an added benefit, antibodies retrieved in this manner are not modified in the Golgi apparatus, which means they possess high-mannose glycans but not plant-specific xylose and fucose residues (Sriraman et al. 2003). The expression construct is the most important consideration because it controls how the antibody transgene is transcribed, processed, translated and to a certain extent how the protein is modified. Each of these factors can have a critical impact on the final yield, quality and stability of the antibody.

5.2 Post-translational modification

Although the protein synthesis and folding pathways are highly conserved between plants and animals, there are some differences in the capacity for post-translational modification. Plants do not, for example, hydroxylate proline residues in recombinant collagen. There are also various differences in glycan structure: plant-derived recombinant human glycoproteins tend to contain the carbohydrate groups $\beta(1\rightarrow2)$ xylose and $\alpha(1\rightarrow3)$fucose, which are absent in mammals, but generally lack the terminal galactose and sialic acid residues that are found on many native human glycoproteins (Figure 15.4).

Since glycan structures can impact on the solubility, stability, immunogenicity and biological activity of recombinant proteins, the 'humanization' of glycan structures produced in plants has been an important topic of research and debate in the scientific community. There has been considerable interest in modifying the plant glycosylation pathway to humanize the glycan profile of recombinant proteins. Several changes in the pathway are required to produce proteins with typical human glycan structures (Warner 2000; Gomord et al. 2005; Faye et al. 2005). Strategies used include the invitro modification of plant-derived recombinant proteins by purified human $\beta(1,4)$-galactosyltransferase and sialyltansferase enzymes (Blixt et al. 2002) and the expression of human $\beta(1,4)$-galactosyltransferase in transgenic plants to produce recombinant antibodies with galactose-extended glycans (Bakker et al. 2001). In the latter case, 30% of the antibody was galactosylated, similar to the proportion found in hybridoma cells. In vivo sialylation will be more difficult to achieve because plants lack the precursors and metabolic capability to produce this carbohydrate group. A more

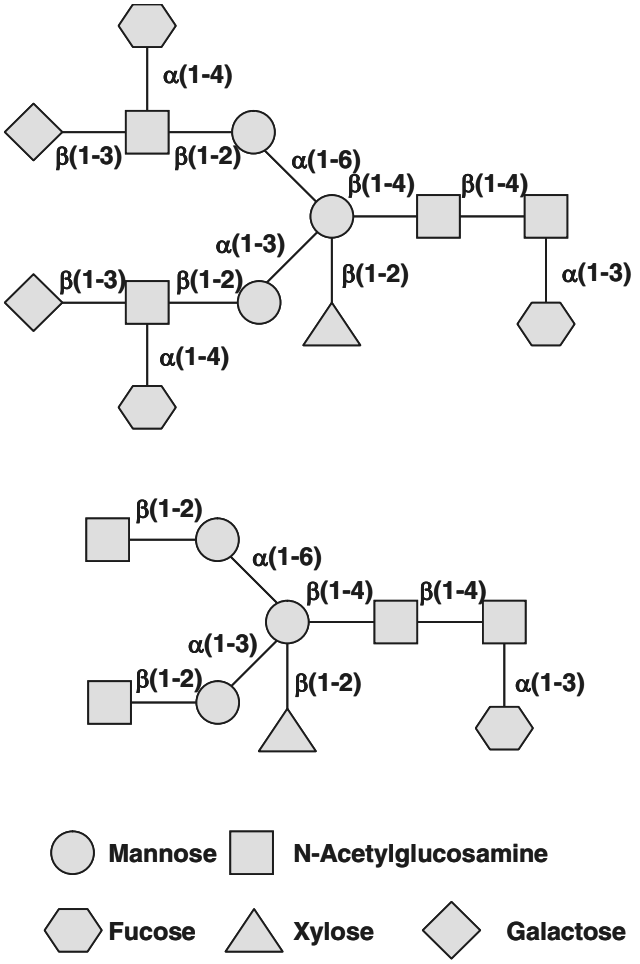


Fig. 15.4. Two glycan structures produced in plants. A) Galactose-extended complex glycan. B) Long chain complex glycan. The xylose and α(1,3) fucose residues are not found in mammals.

recent report documenting sialylation in *Arabidopsis thaliana* suspension cells has been challenged, although the subject remains a matter of controversy (Shah et al. 2003, 2004; Seveno et al. 2004). To remove the non-mammalian β(1→2) xylose and α(1→3)fucose residues, some researchers have explored the possibility of inhibiting the enzymes responsible for synthesizing these groups, while in one case this goal has been achieved in whole *Arabidopsis thaliana* plants by gene knockout techniques

(Strasser et al. 2004). As discussed above, the moss *Physcomitrella patens* can also be modified by gene targeting to eliminate these enzymes (Decker and Reski 2004). Another approach is to prevent the glycoproteins passing through the Golgi, so only high-mannose glycans are added. This can be achieved simply by adding a KDEL C-terminal tag to the antibody, as demonstrated by Sriraman et al. (2004) and Triguero et al. (2005). This issue has been reviewed by Gomord et al. (2004).

5.3 Downstream processing

Downstream processing, the isolation and purification of the recombinant product, is an integral part of every biomanufacturing process. Whichever production system is used, downstream processing represents up to 80% of overall production costs, although this depends on the required level of purity and is highest for clinical-grade materials (Drossard 2003). In many cases, it is necessary to develop specific processing steps for each product, although certain classes of product can be isolated using a standardized approach (e.g. affinity chromatography to isolate recombinant antibodies (Stoger et al. 2005b)). Several aspects of downstream processing have to be customized specifically for plant systems, including the removal of fibres, oils and other by-products from certain crops, and process optimization for the treatment of different plant species and tissues (Menkhaus et al. 2004; Nikolov and Woodard 2004).

For the production of clinical grade antibodies, downstream processing steps need to meet the standards that have been set for other biopharmaceutical production systems, including a strict regime of quality assurance and quality control to achieve approval of regulatory agencies (Fahrner et al. 2001). The initial stages of processing display the greatest variability and have to be optimized in a system-specific manner. Disruption of cell walls and membranes is the first post-harvesting step, but different tissue types (leaves, seeds, fruits etc.) require different forms of treatments (grinding, milling, etc.). After cell disruption, clarification of the extract is often carried out by dead-end or cross-flow filtration, sometimes preceded by bulk cell mass removal using a decanter, plate separator or centrifuge.

Several liquid chromatography steps are required in a full purification protocol, and the initial chromatographic steps require the most specialization for plant-based production. In industrial processing, robust and inexpensive chromatography media are used in the initial steps, accepting that there will be some loss of selectivity and resolution (Bai and Glatz 2003; Menkhaus and Glatz 2005). However, important exceptions include the use of Protein A or Protein G affinity chromatography for antibody purification, and the use of affinity tags and their respective capture agents

(e.g. His_6 and Ni-NTA resin) which are highly selective initial capturing methods.

5.4 Regulatory landscape

One of the greatest uncertainties surrounding the use of plants for the production of pharmaceuticals is the regulatory landscape. While plants are grown in glasshouses and in enclosed bioreactors, the production of pharmaceuticals is regulated in the same way for other production systems, and comes under the authority of the FDA and equivalent agencies in other parts of the world. The switch to open-field conditions adds another layer of regulatory complexity, because the transgenic plants then come under the authority of APHIS (part of the USDA) or their counterparts in Europe and other regions. The involvement of multiple regulatory agencies makes the production process more complex because the extent of each authority's jurisdiction is not always clear, and at the current time only draft guidelines are available (FDA 2002; CPMP 2002). The impact of this is to suppress the market. It is likely that more companies will become interested in plant-derived pharmaceuticals when full guidelines become available, hopefully sometime in mid-2006.

All recombinant pharmaceuticals, including those derived from plants, need to comply with the national and international GMP standards for product safety, quality, potency and efficacy. However, it is not clear at which stage GMP requirements should come into effect when plants are used as the production system, since the strict rules governing defined growth conditions are difficult to implement in the field, where variables such as the weather, differences in soil quality and the presence of other organisms needs to be considered. This is increasingly important now that European regulatory requirements regarding GMP-compliance for the manufacture of medicinal products have extended to the production of clinical trial material (Directive 2001/20/EC).

6. Conclusions

The production of recombinant pharmaceuticals in plants is advantageous, theoretically offering unlimited production scales at unprecedented low manufacturing costs. We are beginning to overcome the technical limitations such as low yields, instability and non-authentic glycan structures that erect obstacles in the path towards commercialization, but more needs to be done to convince industry that plants represent a true alternative to CHO cells and bacteria. Despite the further limitations of a formative and,

in some cases, restrictive regulatory framework, the potential of molecular farming can be seen in the rich IP landscape and the multiple cross-licensing and collaborative ventures that are possible between companies developing production platforms, extraction and separation technologies and those with experience in the latter stages of drug development and marketing. The welcome announcement of the first approved plant-derived veterinary vaccine may open the way for antibodies, and in particular antibodies with therapeutic and diagnostic potential, to follow.

References

Andersen DC, Krummen L (2003) Recombinant protein expression for therapeutic applications. Curr Opin Biotechnol 3: 117-123

Artsaenko O, Kettig B, Fiedler U, Conrad U, Düring K (1998) Potato tubers as a biofactory for recombinant antibodies. Mol Breeding 4: 313-319

Bai Y, Glatz CE (2003) Bioprocess considerations for expanded-bed chromatography of crude canola extract: sample preparation and adsorbent reuse. Biotechnol Bioeng 81: 775-782

Bakker H, Bardor M, Molthoff JW, Gomord V, Elbers I, Stevens LH, Jordi W, Lommen A, Faye L, Lerouge P, Bosch D (2001) Galactose-extended glycans of antibodies produced by transgenic plants. Proc Natl Acad Sci USA 98: 2899-2904

Baneyx F, Mujacic M (2004) Recombinant protein folding and misfolding in *Escherichia coli*. Nature Biotechnol 22: 1399-1408

Benvenuto E, Ordas RJ, Tavazza R, Ancora G, Biocca S, Cattaneo A, Galeffi P (1991) 'Phytoantibodies': a general vector for the expression of immunoglobulin domains in transgenic plants. Plant Mol Biol 17: 865-874

Blixt O, Allin K, Pereira L, Datta A, Paulson JC (2002) Efficient chemoenzymatic synthesis of O-linked sialyl oligosaccharides. J Am Chem Soc 124: 5739-5746

Borisjuk NV, Borisjuk LG, Logendra S, Petersen F, Gleba Y, Raskin I (1999) Production of recombinant proteins in plant root exudates. Nature Biotechnol 17: 466-469

Bouquin T, Thomsen M, Nielsen LK, Green TH, Mundy J, Hanefeld Dziegiel M (2002) Human anti-rhesus D IgG1 antibody produced in transgenic plants. Transgenic Res 11: 115-122

Bruyns AM, De Jaeger G, De Neve M, De Wilde C, Van Montagu M, Depicker A (1996) Bacterial and plant-produced scFv proteins have similar antigen-binding properties. FEBS Lett 386: 5-10

Cabanes-Macheteau M, Fitchette-Laine AC, Loutelier-Bourhis C, Lange C, Vine N, Ma J, Lerouge P, Faye L (1999) N-Glycosylation of a mouse IgG expressed in transgenic tobacco plants. Glycobiology 9: 365-372

Canizares MC, Nicholson L, Lomonossoff GP (2005) Use of viral vectors for vaccine production in plants. Immunol Cell Biol 83: 263-270

Chadd HE, Chamow SM (2001) Therapeutic antibody expression technology. Curr Opin Biotrechnol 12: 188-194

Chargelegue D, Drake PM, Obregon P, Prada A, Fairweather N, Ma JK (2005) Highly immunogenic and protective recombinant vaccine candidate expressed in transgenic plants. Infect Immun 73: 5915-5922

Christensen AH, Quail PH (1996) Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. Transgenic Res 5: 213-218

Christou P, Stoger E, Twyman RM (2004) Monocot systems for molecular farming. In: Fischer R, Schillberg S (eds) *Molecular Farming: Plant-made Pharmaceuticals and Technical Proteins*. John Wiley & Sons Inc., NY, pp 55-67

Chu L, Robinson DK (2001) Industrial choices for protein production by large-scale cell culture. Curr Opin Biotechnol 12: 180-187

Commandeur U, Twyman RM, Fischer R (2003) The biosafety of molecular farming in plants. AgBiotechNet 5: ABN 110

Conrad U, Fiedler U (1998) Compartment-specific accumulation of recombinant immunoglobulins in plant cells: an essential tool for antibody production and immunomodulation of physiological functions and pathogen activity. Plant Mol Biol 38: 101-109

CPMP (2002) Points to consider on quality aspects of medicinal products containing active substances produced by stable transgene expression in higher plants (CPMP/BWP/764/02), The European Agency for the Evaluation of Medicinal Products (EMEA)

Cramer CL, Boothe JG, Oishi KK (1999) Transgenic plants for therapeutic proteins: linking upstream and downstream technologies. Curr Top Microbiol & Immunol 240: 95-118

D'Aoust MA, Lerouge P, Busse U, Bilodeau P, Trepanier S, Gomord V, Faye L, Vezina LP (2004) Efficient and reliable production of pharmaceuticals in alfalfa. In: *Molecular Farming: Plant-made Pharmaceuticals and Technical Proteins* (Fischer R, Schillberg S (eds). John Wiley & Sons, NY, 1-12

Daniell H, Chebolu S, Kumar S, Singleton M, Falconer R (2005a) Chloroplast-derived vaccine antigens and other therapeutic proteins. Vaccine 23: 1779-1783

Daniell H, Kumar S, Dufourmantel N (2005b) Breakthrough in chloroplast genetic engineering of agronomically important crops. Trends Biotechnol 23: 238-245

De Jaeger G, Scheffer S, Jacobs A, Zambre M, Zobell O, Goossens A, Depicker A, Angenon G (2002) Boosting heterologous protein production in transgenic dicotyledonous seeds using *Phaseolus vulgaris* regulatory sequences. Nature Biotechnol 20: 1265-1268

De Neve M, De Loose M, Jacobs A, Van Houdt H, Kaluza B, Weidle U, Van Montagu M, Depicker A (1993) Assembly of an antibody and its derived antibody fragment in *Nicotiana* and *Arabidopsis*. *Transgenic Res* 2: 227-237

De Wilde C, De Rycke R, Beeckman T, De Neve M, Van Montagu M, Engler G, Depicker A (1998) Accumulation pattern of IgG antibodies and Fab fragments in transgenic Arabidopsis thaliana plants. Plant Cell Physiol 39: 639-646

De Wilde C, Peeters K, Jacobs A, Peck I, Depicker A (2002) Expression of antibodies and Fab fragments in transgenic potato plants: a case study for bulk production in crop plants. Mol Breeding 9: 271-282

Decker EL, Reski R (2004) The moss bioreactor. Curr Opin Plant Biol 7: 166-170

Donson J, Kearney CM, Hilf ME, Dawson WO (1991) Systemic expression of a bacterial gene by a tobacco mosaic virus-based vector. Proc Natl Acad Sci USA 88: 7204-7208

Doran PM (2006) Loss of recombinant proteins from plant cell cultures. Trends Biotechnol (in press)

Drake PM, Chargelegue DM, Vine ND, van Dolleweerd CJ, Obregon P, Ma JK (2003) Rhizosecretion of a monoclonal antibody protein complex from transgenic tobacco roots. Plant Mol Biol 52: 233-241

Drossard J (2003) Downstream processing of plant-derived recombinant therapeutic proteins. In: Fischer R, Schillberg S (eds) *Molecular Farming: Plant-made Pharmaceuticals and Technical Proteins*. John Wiley & Sons Inc., NY, pp 217-231

Dyck MK, Lacroix D, Pothier F, Sirard MA (2003) Making recombinant proteins in animals – different systems, different applications. Trends Biotechnol 21: 394-399

Ehsani P, Meunier A, Nato F, Jafari A, Nato A, Lafaye P (2003) Expression of anti human IL-4 and IL-6 scFvs in transgenic tobacco plants. Plant Mol Biol 52: 17-29

Fahrner RL, Knudsen HL, Basey CD, Galan W, Feuerhelm D, Vanderlaan M, Blank GS (2001) Industrial purification of pharmaceutical antibodies: development, operation, and validation of chromatography processes. Biotechnol Genet Eng Rev 18: 301–327

Faye L, Boulaflous A, Benchabane M, Gomord V, Michaud D (2005) Protein modifications in the plant secretory pathway: current status and practical implications in molecular pharming. Vaccine 23: 1770-1778

FDA (2002) Guidance for industry. Drugs, biologics, and medical devices derived from bioengineered plants for use in humans and animals. Food and Drug Administration

Fischer R, Emans N, Schuster F, Hellwig S, Drossard J (1999) Towards molecular farming in the future: using plant-cell-suspension cultures as bioreactors. Biotechnol Appl Biochem 30: 109–112

Fischer R, Emans N (2000) Molecular farming of pharmaceutical proteins. Transgenic Res 9: 279-299

Francisco JA, Gawlak SL, Miller M, Bathe J, Russell D, Chace D, Mixan B, Zhao L, Fell HP, Siegall CB (1997) Expression and characterization of bryodin 1 and a bryodin 1-based single-chain immunotoxin from tobacco cell culture. Bioconjug Chem 8: 708-713

Franconi R, Roggero P, Pirazzi P, Arias FJ, Desiderio A, Bitti O, Pashkoulov D, Mattei B, Bracci L, Masenga V, Milne RG, Benvenuto E (1999) Functional expression in bacteria and plants of an scFv antibody fragment against tospoviruses. Immunotechnology 4: 189-201

Franklin SE, Mayfield SP (2005) Recent developments in the production of human therapeutic proteins in eukaryotic algae. Expert Opin Biol Ther 5: 225-235

Gasdaska JR, Spencer D, Dickey L (2003) Advantages of therapeutic protein production in the aquatic plant Lemna. BioProcessing J Mar/Apr: 49-56

Gavilondo JV, Larrick JW (2000) Antibody production technology in the millennium. Biotechniques 29: 128-145

Gerngross TU (2004) Advances in the production of human therapeutic proteins in yeasts and filamentous fungi. Nature Biotechnol 22: 1409-1414

Gleba Y, Marillonnet S, Klimyuk V (2004) Engineering viral expression vectors for plants: the 'full virus' and the 'deconstructed virus' strategies. Curr Opin Plant Biol 7: 182-188

Gleba Y, Klimyuk V, Marillonnet S (2005) Magnifection – a new platform for expressing recombinant vaccines in plants. Vaccine 23: 2042-2048

Gomord V, Sourrouille C, Fitchette AC, Bardor M, Pagny S, Lerouge P, Faye L (2004) Production and glycosylation of plant-made pharmaceuticals: the antibodies as a challenge. Plant Biotechnol J 2: 83-100

Gomord V, Chamberlain P, Jefferis R, Faye L (2005) Biopharmaceutical production in plants: problems, solutions and opportunities. Trends Biotechnol 23: 559-565

Green L (1999) Antibody engineering via genetic engineering of the mouse: xenomouse strains are a vehicle for the facile generation of therapeutic human monoclonal antibodies. J Immunol Methods 231: 11-23

Griffiths A, Duncan A (1998) Strategies for selection of antibodies by phage display. Curr Opin Biotechnol 9: 102-108

Harvey AJ, Speksnijder G, Baugh LR, Morris JA, Ivarie R (2002) Expression of exogenous protein in the egg white of transgenic chickens. Nature Biotechnol 20: 396–399

Hellwig S, Drossard J, Twyman RM, Fischer R (2004) Plant cell cultures for the production of recombinant proteins. Nature Biotechnol 22: 1415-1422

Hendy S, Chen ZC, Barker H, Santa Cruz S, Chapman S, Torrance L, Cockburn W, Whitelam GC (1999) Rapid production of single-chain Fv fragments in plants using a potato virus X episomal vector. J Immunol Methods 231: 137-146

Hiatt A, Cafferkey R, Bowdish K (1989) Production of antibodies in transgenic plants. Nature 342: 76-78

Hood EE, Woodard SL, Horn ME (2002) Monoclonal antibody manufacturing in transgenic plants — myths and realities. Curr Opin Biotechnol 13: 630–635

Hull AK, Criscuolo CJ, Mett V, Groen H, Steeman W, Westra H, Chapman G, Legutki B, Baillie L, Yusibov V (2005) Human-derived, plant-produced monoclonal antibody for the treatment of anthrax. Vaccine 23: 2082-2086

Ikonomou L, Schneider YJ, Agathos SN (2003) Insect cell culture for industrial production of recombinant proteins. Appl Microbiol Biotechnol 62: 1-20

Jefferis R (2001) Glycosylation of human IgG antibodies: relevance to therapeutic applications. Biopharm 2001: 19-27

Kapila J, De Rycke R, van Montagu M, Angenon G (1997) An *Agrobacterium*-mediated transient gene expression system for intact leaves. Plant Sci 122: 101–108

Kathuria S, Sriraman R, Nath R, Sack M, Pal R, Artsaenko O, Talwar GP, Fischer R, Finnern R (2002) Efficacy of plant-produced recombinant antibodies against HCG. Hum Reprod 17: 2054-2061

Khoudi H, Laberge S, Ferullo JM, Bazin R, Darveau A, Castonguay Y, Allard G, Lemieux R, Vezina LP (1999) Production of a diagnostic monoclonal antibody in perennial alfalfa plants. Biotechnol Bioeng 64: 135–143

Kipriyanov SM, Little M (1999) Generation of recombinant antibodies. Mol Biotechnol 12: 173-201

Ko K, Tekoah Y, Rudd PM, Harvey DJ, Dwek RA, Spitsin S, Hanlon CA, Rupprecht C, Dietzschold B, Golovkin M, Koprowski H (2003) Function and glycosylation of plant-derived antiviral monoclonal antibody. Proc Natl Acad Sci USA 100: 8013-8018

Ko K, Steplewski Z, Glogowska M, Koprowski H (2005) Inhibition of tumor growth by plant-derived mAb. Proc Natl Acad Sci USA 102: 7026-7030

Komarnytsky S, Borisjuk NV, Borisjuk LG, Alam MZ, Raskin I (2000) Production of recombinant proteins in tobacco guttation fluid. Plant Physiol 124: 927-933

Lelivelt CL, McCabe MS, Newell CA, Desnoo CB, van Dun KM, Birch-Machin I, Gray JC, Mills KH, Nugent JM (2005) Stable plastid transformation in lettuce (*Lactuca sativa* L.) Plant Mol Biol 58: 763-774

Ma JK, Hiatt A, Hein M, Vine ND, Wang F, Stabila P, van Dolleweerd C, Mostov K, Lehner T (1995) Generation and assembly of secretory antibodies in plants. Science 268: 716-719

Ma JK, Drake PM, Christou P (2003) The production of recombinant pharmaceutical proteins in plants. Nat Rev Genet 4: 794-805

Magee AM, Coyne S, Murphy D, Horvath EM, Medgyesy P, Kavanagh TA (2004) T7 RNA polymerase-directed expression of an antibody fragment transgene in plastids causes a semi-lethal pale-green seedling phenotype. Transgenic Res 13: 325-337

Makvandi-Nejad S, McLean MD, Hirama T, Almquist KC, Mackenzie CR, Hall JC (2005) Transgenic tobacco plants expressing a dimeric single-chain variable fragment (scfv) antibody against *Salmonella enterica* serotype Paratyphi B. Transgenic Res 14: 785-792

Maliga P (2003) Progress towards commercialization of plastid transformation technology. Trends Biotechnol 21: 20-28

Maliga P (2004) Plastid transformation in higher plants. Annu Rev Plant Biol 55: 289-313

Marillonnet S, Thoeringer C, Kandzia R, Klimyuk V, Gleba Y (2005) Systemic *Agrobacterium tumefaciens*-mediated transfection of viral replicons for efficient transient expression in plants. Nature Biotechnol 23: 718-723

Mayfield SP, Franklin SE, Lerner RA (2003) Expression and assembly of a fully active antibody in algae. Proc Natl Acad Sci USA 100: 438-442

Mayfield SP, Franklin SE (2005) Expression of human antibodies in eukaryotic micro-algae. Vaccine 23: 1828-1832

McCormick AA, Kumagai MH, Hanley K, Turpen TH, Hakim I, Grill LK, Tuse D, Levy S, Levy R (1999) Rapid production of specific vaccines for lymphoma by expression of the tumor-derived single-chain Fv epitopes in tobacco plants. Proc Natl Acad Sci USA 96: 703-708

Menkhaus TJ, Bai Y, Zhang C, Nikolov ZL, Glatz CE (2004) Considerations for the recovery of recombinant proteins from plants. Biotechnol Prog. 20: 1001-1014

Menkhaus TJ, Glatz CE (2005) Antibody capture from corn endosperm extracts by packed bed and expanded bed adsorption. Biotechnol Prog. 21: 473-485

Nikolov ZL, Woodard SL (2004) Downstream processing of recombinant proteins from transgenic feedstock. Curr Opin Biotechnol 15: 479-486

Nugent GD, Coyne S, Nguyen TT, Kavanagh TA, Dix PJ (2006) Nuclear and plastid transformation of *Brassica oleracea* var. botrytis (cauliflower) using PEG-mediated uptake of DNA into protoplasts. Plant Sci 170: 135-142

Padidam M (2003) Chemically regulated gene expression in plants. Curr Opin Plant Biol 6: 169–177

Perrin Y, Vaquero C, Gerrard I, Sack M, Drossard J, Stoger E, Christou P, Fischer R (2000) Transgenic pea seeds as bioreactors for the production of a single-chain Fv fragment (scFV) antibody used in cancer diagnosis and therapy. Mol Breeding 6: 345-352

Raju TS, Briggs J, Borge SM, Jones AJS (2000) Species-specific variation in glycosylation of IgG: evidence for the species-specific sialylation and branch-specific galactosylation and importance for engineering recombinant glycoprotein therapeutics. Glycobiolgy 10: 477-486

Rodriguez M, Ramirez NI, Ayala M, Freyre F, Perez L, Triguero A, Mateo C, Selman-Housein G, Gavilondo JV, Pujol M (2005) Transient expression in tobacco leaves of an aglycosylated recombinant antibody against the epidermal growth factor receptor. Biotechnol Bioeng 89: 188-194

Schaefer DG (2002) A new moss genetics: targeted mutagenesis in *Physcomitrella patens*. Annu Rev Plant Biol 53: 477-501

Schillberg S, Zimmermann S, Voss A, Fischer R (1999) Apoplastic and cytosolic expression of full-size antibodies and antibody fragments in *Nicotiana tabacum*. Transgenic Res 8: 255-263

Schillberg S, Zimmermann S, Zhang MY, Fischer R (2001) Antibody-based resistance to plant pathogens. Transgenic Res 10: 1-12

Schillberg S, Emans N, Fischer R (2002) Antibody molecular farming in plants and plant cells. Phytochem Rev 1: 45-54

Schillberg S, Fischer R, Emans N (2003) Molecular farming of recombinant antibodies in plants. Cell Mol Life Sci 60: 433-445

Schunmann PHD, Coia G, Waterhouse PM (2002) Biopharming the SimpliRED™ HIV diagnostic reagent in barley, potato and tobacco. Mol. Breeding 9: 113-121

Schunmann PHD, Surin B, Waterhouse PM (2003) A suite of novel promoters and terminators for plant biotechnology. II. The pPLEX series for use in monocots Funct Plant Biol 30: 453-460

Semenyuk EG, Orlova IV, Stremovskii OA, Balandin TG, Nosov AM, Bur'yanov Y, Deev SM (2002) Transgenic tobacco plants produce miniantibodies against human ferritin. Dokl Biochem Biophys 384: 176-178

Seveno M, Bardor M, Paccalet T, Gomord V, Lerouge P, Faye L (2004) Glycoprotein sialylation in plants? Nature Biotechnol 22: 1351-1352

Shah MM, Fujiyama K, Flynn CR, Joshi L (2003) Sialylated endogenous glycoconjugates in plant cells. Nature Biotechnol 21: 1470-1471

Shah MM, Fujiyama K, Flynn CR, Joshi L (2004) Glycoprotein sialylation in plants? Reply. Nature Biotechnol 22: 1352-1353

Sharp JM, Doran PM (2001a) Characterization of monoclonal antibody fragments produced by plant cells. Biotechnol Bioeng 73: 338-346

Sharp JM, Doran PM (2001b) Strategies for enhancing monoclonal antibody accumulation in plant cell and organ cultures. Biotechnol Prog 17: 979-992

Sidhu SS (2000) Phage display in pharmaceutical biotechnology. Curr Opin Biotechnol 11: 610-616

Sriraman R, Sack M, Talwar GP, Fischer R (2003) Glycosylation of recombinant antibodies in plants. In: Proceedings of the Ninth Annual Ranbaxy Science Foundation Symposium. Ranbaxy Science Foundation, New Delhi, pp 89-98

Sriraman R, Bardor M, Sack M, Vaquero C, Faye L, Fischer R, Finnern R, Lerouge P (2004) Recombinant anti-hCG antibodies retained in the endoplasmic reticulum of transformed plants lack core-xylose and core- (1,3)-fucose residues. Plant Biotechnol J 2: 279-287

Stoger E, Vaquero C, Torres E, Sack M, Nicholson L, Drossard J, Williams S, Keen D, Perrin Y, Christou P, Fischer R (2000) Cereal crops as viable production and storage systems for pharmaceutical scFv antibodies. Plant Mol Biol 42: 583-590

Stoger E, Sack M, Perrin Y, Vaquero C, Torres E, Twyman RM, Christou P, Fischer R (2002) Practical considerations for pharmaceutical antibody production in different crop systems. Mol Breeding 9: 149-158

Stoger E, Ma JK, Fischer R, Christou P (2005a) Sowing the seeds of success: pharmaceutical proteins from plants. Curr Opin Biotechnol 16: 167-173

Stoger E, Sack M, Nicholson L, Fischer R, Christou P (2005b) Recent progress in plantibody technology. Curr Pharm Des 11: 2439-2457

Strasser R, Altmann F, Mach L, Glossl J, Steinkellner H (2004) Generation of Arabidopsis thaliana plants with complex N-glycans lacking beta1,2-linked xylose and core alpha1,3-linked fucose. FEBS Lett 561: 132-136

Subroto MA, Hamill JD, Doran PM (1996) Development of shooty teratomas from several solanaceous plants: growth kinetics, stoichiometry and alkaloid production. J Biotechnol 45: 45-57

Torres E, Vaquero C, Nicholson L, Sack M, Stoger E, Drossard J, Christou P, Fischer R, Perrin Y (1999) Rice cell culture as an alternative production system for functional diagnostic and therapeutic antibodies. Transgenic Res 8: 441-449

Triguero A, Cabrera G, Cremata JA, Yuen CT, Wheeler J, Ramírez NI (2005) Plant-derived mouse IgG monoclonal antibody fused to KDEL endoplasmic reticulum-retention signal is N-glycosylated homogeneously throughout the plant with mostly high-mannose-type N-glycans. Plant Biotechnol J 3: 449-457

Twyman RM, Stoger E, Schillberg S, Christou P, Fischer R (2003) Molecular farming in plants: host systems and expression technology. Trends Biotechnol 21: 570-578

Twyman RM, Schillberg S, Fischer R (2005) Transgenic plants in the biopharmaceutical market. Expert Opin Emerg Drugs 10: 185-218

Valdes R, Gomez L, Padilla S, Brito J, Reyes B, Alvarez T, Mendoza O, Herrera O, Ferro W, Pujol M, Leal V, Linares M, Hevia Y, Garcia C, Mila L, Garcia O, Sanchez R, Acosta A, Geada D, Paez R, Luis Vega J, Borroto C (2003a) Large-scale purification of an antibody directed against hepatitis B surface antigen from transgenic tobacco plants. Biochem Biophys Res Commun 308: 94-100

Valdes R, Reyes B, Alvarez T, Garcia J, Montero JA, Figueroa A, Gomez L, Padilla S, Geada D, Abrahantes MC, Dorta L, Fernandez D, Mendoza O, Ramirez N, Rodriguez M, Pujol M, Borroto C, Brito J (2003b) Hepatitis B surface antigen immunopurification using a plant-derived specific antibody produced in large scale. Biochem Biophys Res Commun 310: 742-747

Vaquero C, Sack M, Chandler J, Drossard J, Schuster F, Monecke M, Schillberg S, Fischer R (1999) Transient expression of a tumor-specific single-chain fragment and a chimeric antibody in tobacco leaves. Proc Natl Acad Sci USA 96: 11128-11133

Vaquero C, Sack M, Schuster F, Finnern R, Drossard J, Schumann D, Reimann A, Fischer R (2002) A carcinoembryonic antigen-specific diabody produced in tobacco. FASEB J 16: 408-410

Verch T, Yusibov V, Koprowski H (1998) Expression and assembly of a full-length monoclonal antibody in plants using a plant virus vector. J Immunol Methods 220: 69-75

Vine ND, Drake P, Hiatt A, Ma JK (2001) Assembly and plasma membrane targeting of recombinant immunoglobulin chains in plants with a murine immunoglobulin transmembrane sequence. Plant Mol Biol 45: 159-167

Warner TG (2000) Metabolic engineering glycosylation: biotechnology's challenge to the glycobiologist in the new millennium. In: Carbohydrates in Chemistry and Biology (Ernst B, Hart GW, Sanay P (eds), Wiley-VCH, NY, pp 1043-1064

Wurm FM (2004) Production of recombinant protein therapeutics in cultivated mammalian cells. Nature Biotechnol 22: 1393-1398

Yano A, Maeda F, Takekoshi M (2004) Transgenic tobacco cells producing the human monoclonal antibody to hepatitis B virus surface antigen. J Med Virol 73: 208-215

Yusibov V, Rabindran S, Commandeur U, Twyman RM, Fischer R (2006) The potential of plant virus vectors for vaccine production. Drugs in R&D (in press)

Zeitlin L, Olmsted SS, Moench TR, Co MS, Martinell BJ, Paradkar VM, Russell DR, Queen C, Cone RA, Whaley KJ (1998) A humanized monoclonal antibody produced in transgenic plants for immunoprotection of the vagina against genital herpes. Nature Biotechnol 16: 1361–1364

Ziegler A, Cowan GH, Torrance L, Ross HA, Davies HV (2000) Facile assessment of cDNA constructs for expression of functional antibodies in plants using the potato virus X vector. Mol Breeding 6: 327-335

Zimmermann S, Schillberg S, Liao YC, Fisher R (1998) Intracellular expression of TMV-specific single-chain Fv fragments leads to improved virus resistance in *Nicotiana tabacum*. Mol Breeding 4: 369–379

16

Molecular tailoring and boosting of bioactive secondary metabolites in medicinal plants

Antonella Leone [1], Stefania Grillo[2], Luigi Monti[3], Teodoro Cardi[2]

[1] Department of Pharmaceutical Sciences, Faculty of Pharmacy, University of Salerno, Italy [2] CNR-IGV, Institute of Plant Genetics, Portici Division, National Research Council, Portici, Italy [3] Department of Soil, Plant and Environmental Sciences, University of Naples 'Federico II', Portici, Italy

1. Introduction

Although the production of most of the current medicines is based on chemical synthesis, more than 25% of the current prescribed drugs contains at least one active ingredient of plant origin (Kaufman et al 1999). Examples of important plant-derived pharmaceuticals include the anti-tumoral taxol and vinblastine, the antimalarial drug quinine and artemisinin, the analgesical morphine and codeine. In addition, it has been estimated that more than 80% of the world's population in developing countries depends primarily on herbal medicine for basic healthcare needs (Vines 2004). There is also a revival of traditional medicine in developed countries and an increase in the use of herbal remedies. The world market of herbal medicines, including herbal and raw material, has been estimated to have an annual growth rate between 5-15%. Total global herbal drug market is estimated as US $ 62 billion and it is expected to grow to US $ 5 trillion by the year 2050 (Joshi et al. 2004). At same time, there is a growing concern on loss of genetic diversity since about 75% of the 50,000 different medicinal plant species in use are collected from the wild (Edwards 2004). Moreover, to rely solely on wild spontaneous plants as a

P. Ranalli (ed.), Improvement of Crop Plants for Industrial End Uses, 471–507.

production system can be extremely dangerous, as shown recently by severe shortage problems of the antimalarial artemisinin (Scheindlin 2005). Additionally, bioactive plant compounds are produced generally at very low amount and, often, it is not economically convenient to extract them from natural sources.

Altogether these considerations have opened the way to a new renaissance in the field of genetic and metabolic engineering studies to modify biosynthetic pathways in medicinal plants and to enhance the production of bioactive phytopharmaceuticals.

Far to be exhaustive, the present review is aimed at presenting an updated view of the most recent advances in engineering tailored secondary metabolites in medicinal plants. Analysis and discussion will be restricted to the successes, the pitfalls, the bottlenecks and the evolution in the strategies that have been used or might be used to boost the synthesis of plant compounds that exert specific pharmacological actions and that may be used for specific health problems over short- or long-term intervals. Engineering of plant compounds, serving nutritional or health benefits arising from long-term use as food (nutraceuticals), will be not covered in the present review.

2. Evolution of diversity of secondary metabolites

Most of the plant compounds exerting pharmacological activity belongs to the class of small molecules (< 1000 Da), known collectively as secondary metabolites. To date, more than 100,000 different plant metabolites have been described, that represent less than 10% of the actual total present in nature (Wink 1999). The astonishing chemical and structural heterogeneity of plant secondary metabolites is the result of the increased evolutionary plant adaptability to an unstable and challenging environment. Besides their role in the plant's defence, secondary metabolites have also potential pharmacological effects in humans. In general terms, to promote plant survival under biotic and abiotic stress, structures of secondary metabolites have evolved to interact with molecular targets affecting cellular and physiological functions in competing microorganisms, plants and animals. In this respect, some plant secondary metabolites may exert their actions by resembling endogenous metabolites, ligands, hormones, signal transduction molecules, or neurotransmitters, thus having beneficial medicinal effects on humans due to the similarities in their potential target sites (*e.g.* central nervous system, endocrine system etc) (Wink and Schimmer 1999). For example, plant secondary products that are cytotoxic

towards plant pathogens could be useful also as antimicrobial medicines in humans, if not too toxic. Similarly, secondary metabolites active against plant herbivores, due to their neurotoxical activity, could prove to be beneficial in relieving disturbs related to the central nervous system in humans (i.e. as antidepressants, sedatives, muscle relaxants, or anaesthetics).

Much of the chemical and structural diversity of plant secondary metabolites derives from a limited number of backbone structures. As an example, the more than 40,000 different molecules ascribed to the class of isoprenoids, are all synthesized through the condensation of isopentyl diphosphate (IPP) and its allylic isomer, methylallyl diphosphate (DMAPP) (Croteau et al. 2000) and most alkaloids are derived through decarboxylation of amino-acid precursors (i.e. ornithine, lysine, tryptophan and histidine) (Facchini 2001). It is only the subsequent decoration of these common precursors, by addition of functional groups and/or modification operated by quite conserved enzymatic activities (e.g. hydroxylation, methylation, acetylation, glycosilation etc), that generates the amazing diversity of plant secondary metabolites and that imparts a genus- or species-specific chemical signature. Each biosynthetic route of secondary metabolism derives from primary metabolism and involves complex and often specific enzymatic reactions that lead to specific biosynthetic pathways to yield different classes of natural products. In many cases, the first reaction leading to the synthesis of a new secondary metabolite is pivotal to the formation of a new secondary biosynthetic pathway. If the resultant new encoded enzyme can form a secondary product that is advantageous for plant survival, the trait is inherited under selection pressure. In fact, there is increasing evidence that duplication of essential genes of primary metabolism is an important evolutionary mechanism for gene recruitment in secondary metabolism. During evolution, these duplicated genes acquired new functions and were optimized and diversified for their role in new pathways (Pickersky and Gang 2000; Noel et al. 2005; Pickersky et al. 2006). As it will discuss later, this feature implies that different strategies of genetic and metabolic engineering might be adopted according to which part of the pathway is rate-limiting for the synthesis of a specific bioactive secondary metabolites, i.e. enzymes involved in the synthesis of precursors or in the following decoration.

3. General concepts and molecular tools for metabolic engineering

Recent advances in plant biotechnology, molecular biology and genomics have created promising new opportunities in using plants as efficient, environmentally friendly and renewable chemical factories. The possibility of exploiting the biosynthetic capacity of plants to meet future demands for pharmaceuticals is dependent on a detailed understanding of the biochemical enzymatic reactions involved in secondary metabolites pathways, and on a thorough knowledge on the relative genes and their regulation.

In general terms, development of metabolic engineering strategies to boost the production of bioactive secondary metabolites in *in vivo* plants or in plant tissue cultures requires: i) a systematic expansions of the available molecular toolbox (i.e. cloned genes for enzymes involved in a specific pathway and regulatory genes), ii) detailed and accurate knowledge on the biochemistry of the metabolic pathways under study, including rate-limiting enzymatic reactions, on the cellular compartmentation of the specific compounds and their catabolism and iii) appropriate and efficient systems for genetic transformation of the plant of interest.

Presently, a major constraint in engineering plant secondary metabolite production is that only few genes of these pathways are known, especially for medicinal plants. Impressive progress has been made for model plants, especially *Arabidopsis thaliana*, for which emerging technologies, genomics, functional genomics, transcriptomics, metabolomics are providing efficient tools to identify biosynthetic and regulatory genes involved in the individual secondary metabolite pathway. Though an increasing number of genes involved in secondary metabolic pathways have been mined from the genome sequence of model plant *A. thaliana* and *Oryza sativa* or other crop plants, this knowledge is of limited values for secondary metabolism of medicinal plants, that is per definition species-specific. For medicinal plants, an attractive alternative to hunt genes involved in the biosynthesis of specific secondary metabolites is offered by random EST sequencing, as reported for *Stevia rebaudiana* (Brandle et al. 2002) and *Ocymum basilicum* (Gang et al. 2001). Sequencing of EST libraries highly enriched in the metabolic genes of interest, such as those from highly specialized cell types, (e.g. glandular trichomes or other specialized tissues) (Gang et al. 2001) or from elicited plant cells or tissues, may serve also as a short-cut to gene discovery in medicinal plants. This last approach has rescued several genes involved in the synthesis of the anticancer drug taxol in a cDNA library from *Taxus*

cuspidata cells induced for taxoid biosynthetic with methyl jasmonate (Jennewein et al. 2004). An ample repertoire of known and novel genes in the tobacco secondary metabolism has been obtained by transcriptional profiling by cDNA-AFLP of elicited plant cells, as reported for jasmonate-elicited tobacco BY-2 cells (Goossens et al. 2003) or *Catharanthus roseus* cells (Rischer et al. 2006).

With only few exceptions, widely used medicinal plants have not received the extensive studies devoted to food crops or model plants, and therefore, for this group of plants there is a poor and scattered characterization of plant secondary metabolites pathway at the level of biosynthetic intermediates and enzymes (Briskin 2000).

Finally, although an efficient transfer delivery system, using either *Agrobacterium rhizogenes* or *A. tumefaciens,* is already available for several important medicinal plants, including *Atropa belladonna*, *Catharanthus roseus, Papaverum somniferum, Artemisia* spp, members of the family *Solanaceae*, *Taraxacum platycarpum*, *Taxus* spp. *Echinacea*, *Scrophularia*, *Digitalis*, *Thalictrum*, *Salvia* spp and others (Bajaj 1999), many medicinal plants are still not prone to genetic transformation, often because of the lack of intensive experimental work. However, in many cases the bottleneck is not genetic transformation *per se* but rather the unfeasibility of *in vitro* organogenesis. This limitation can be overcome by establishing stable transformed cell lines or hairy roots for large-scale production in bio-fermentors.

4. Boosting the metabolic flux towards a target compound

Provided that all the biochemical and genetic information of a metabolic pathway leading to an end-product of interest are available, along with a suitable transformation system for a specific medicinal plant, the choice of the most convenient strategy of metabolic engineering to enhance biosynthesis of one or more secondary metabolites depends on the specific metabolic pathway, but, basically may be achieved through: i) over-expression of a gene encoding an enzyme known to be rate-limiting; ii) down-regulation of a gene encoding the first enzyme of a lateral competitive chain or involved in catabolic reactions; iii) increase of intracellular compartmentation of the interested compound, to avoid feedback control and/or cellular toxicity, and iv) over-expression of multiple genes or coordinate over-expression by regulatory genes (Fig. 16.1).

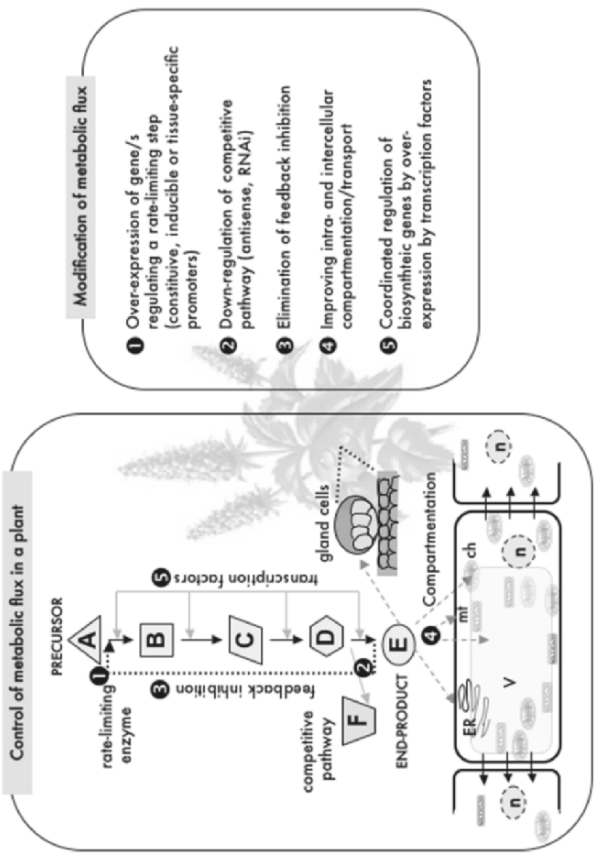


Fig. 16.1. Control of metabolic flux in a plant and possible strategies of metabolic engineering. **n**, nucleus; **mt**, mitochondria; **ch**, chloroplast; **V**, vacuole; **ER**, endoplasmic reticulum.

Pioneer work in engineering metabolically medicinal plants was based on over-expressing one or a few genes thereby overcoming specific rate-limiting steps in the pathway, or to shut down competitive pathways and to decrease catabolism of the product of interest. However, identifying rate-limiting reactions in one specific pathway is often difficult and, besides, the level of the end-product accumulation can be controlled by more than one enzymatic activity. Secondly, attempts have been made to change the expression of regulatory genes that control multiple biosynthetic genes. Recent large-scale studies of gene expression have fuelled the present knowledge on mechanisms that govern transcriptional regulation of complex metabolic pathway. All these strategies will be discussed in the following paragraphs, reporting the most recent results obtained for medicinal plants (Table 16.1).

4.1 One is better than nothing: over-expression of single genes for rate limiting reactions

A typical metabolic engineering approach generally focuses on a particular metabolic intermediate or product. A critical evaluation of the current knowledge on that specific biosynthetic pathway may identify candidates for rate-limiting enzymatic reactions and open the way to a possible strategy of genetic engineering of the identified limiting target gene. As mentioned before, the main constraint for applying this principle in plants, and especially for medicinal ones, is that detailed and unequivocal information on precursors, metabolic intermediates and the relative conversion enzymes are still lacking and, therefore, in many cases, it is virtually unfeasible to establish unambiguously which step is rate-limiting in a specific metabolic pathway. Current up-graded analytical technologies, such as genome sequencing, microarray analysis and sophisticated metabolic profiling, have ameliorated the predictability of metabolic engineering, but still the final effect of manipulating a single gene is under a strict control of the cellular homeostasis. Despite the trial-and error approach adopted principally at the beginning of the metabolic engineering era, the relatively simple way to introduce single genes for rate limiting enzymes, also from

Table 16.1. List of medical plants engineered for the production of secondary metabolites.

Plant species	Target gene	Source	Target compounds	Strategy	Results	References
Atropa belladonna	Hyosciamine 6β-hydroxylase (H6H)	*H. niger*	Tropane alkaloids	Constitutive over-expression in plant	Accumulation of scopolamine	Yun et al. 1992
	Putrescine N-methyl transferase (PMT)	*N. tabacum*	Tropane alkaloids	Constitutive over-expression in plant	No major effects in alkaloid production	Rothe et al. 2003
	Triptone reductase I + II	*D. stramonium*	Tropane alkaloids	Constitutive over-expression in hairy roots	Accumulation of tropine, pseudo- tropine, scopolamine and calystegine	Richter et al. 2005
Cinchona officinalis	Tryptophan decarboxylase (TDC) + Strictosidine synthase (STR)	*C. roseus*	Terpenoid indole alkaloids	Constitutive over-expression in hairy roots	Accumulation of high levels of tryptamine, strictosidine and TIA derivatives quinine and quinidine	Geerlings et al. 1999
Catharanthus roseus	Tryptophan decarboxylase (TDC) + Strictosidine synthase (STR)	Endogenous	Terpenoid indole alkaloids	Constitutive over-expression in cells	Increased accumulation of tryptamine, strictosidine and derivatives in STR transgenic cells; no effects of TDC overexpression	Canel et al. 1998

Plant species	Target gene	Source	Target compounds	Strategy	Results	References
	Tryptophan decarboxylase (TDC)	Endogenous	Terpenoid indole alkaloids	Constitutive over-expression /antisense in call	Increased tryptamine conitent in overexpressing calli; no effects in TIA accumulation	Goddijn et al. 1995 Hughes et al. 2004b
	3-hydroxy-3-methylglutaryl-CoA reductase (HMGR)	Hamster	Isoprenoids/ Terpenoids	Constitutive over-expression in hairy roots	Accumulation of high levels of campesterol and serpentine	Ayora-Talavera et al. 2002
	Anthranilate synthase α subunit (ASα)	*A. thaliana*	Terpenoid indole alkaloids	Inducible over-expression in hairy roots	High extent of tryptophan and triptamineaccumulation; no effect on TIA derivatives	Hughes et al. 2004b
	Anthranilate synthase α subunit (ASα) + Tryptophan decarboxylase (TDC)	*A. thaliana* Endogenous	Terpenoid indole alkaloids	Inducible over-expression in hairy roots	High accumulation of tryptamine; no TIA derivatives accumulation	Hughes et al. 2004a
	Anthranilate synthase β subunit (ASβ) + Anthranilate synthase α subunit (ASα)	*A. thaliana* *A. thaliana*	Terpenoid indole alkaloids	Inducible over-expression in hairy roots	Content of tryptophane and triptamine increased dramatically upon induction; no effect on TIA derivatives	Hong et al. 2006
	Anthranilate synthase β subunit (ASβ) + Anthranilate synthase α subunit (ASα) + Tryptophan decarboxylase (TDC)	*A. thaliana* *A. thaliana* Endogenous	Terpenoid indole alkaloids	Constitutive / inducible over-expression in hairy roots	High increase of tryptophane and triptamine. Changes in TIA accumulation profiles	Hong et al. 2006
	ORCA 3	Endogenous	Terpenoid indole alkaloids	Activation tagging/ constitutive over-expression in cells	Changes in metabolic flux to indole precursors; tryptamine, tryptophan and TIA accumulation	van der Fits and Memelink 2000

Table 16.1. (Continued).

Plant species	Target gene	Source	Target compounds	Strategy	Results	References
Mentha piperita L	Deoxyxylulose phoshate Reducto isomerase (DXR) Menthofuran synthase (MFS)	Endogenous	Monoterpenes	Constitutive over-expression /Co-suppression in plants	Accumulation of essential oils or selective reduction of undesiderable mono-terpenes obtained by DXR over-expression and MFS reduction respectively	Mahmoud and Croteau 2001
	Menthofuran synthase (MFS)	Endogenous	Monoterpenes	Constitutive over- / an-tisense expression in calli	Menthofuran suppression increased accumulation of menthone and menthol. Over-expression led to op-posite results	Mahmoud and Croteau 2003
	Limonene 3-hydroxylase	Endogenous	Monoterpenes	Constitutive over-expression/co-suppression in plants	Accumulation of limonene by co-suppression; no ef-fects by over-expression	Mahmoud and Croteau 2004
	4-S limonene synthase	*M. spicata*	Monoterpenes	Constitutive over-expression in plant	No effect in essential oil accumulation	Mahmoud and Croteau 2004
	4-S limonene synthase	*M. spicata*	Monoterpenes	Constitutive over-expression in plant	Accumulation of total monoterpenes and of intermediate compound pulegone. Same results in *M. arvensis*	Diemer et al. 2001
	4-S limonene synthase	*M. spicata*	Monoterpenes	Constitutive over-expression in plant and protoplasts	Changes in essential oil profile (high menthone and methofuran, low menthol)	Kransnyansky et al. 1999
Papaver somniferum L.	Codeinone reductase (COR)	Endogenous	Alkaloids	Down regulation by RNAi in plant	Accumulation of non-narcotic alkaloid reticuline	Allen et al. 2004
	Berberine Bridge Enzyme (BBE)	Endogenous	Benzylsoquinoline alkaloids	Constitutive antisense expression in plant	Changes in intermediate alkaloid pathways. Major change in alkaloid profiles occurred in latex	Frick et al. 2004

Plant species	Target gene	Source	Target compounds	Strategy	Results	References
Eschscholzia californica Cham	Berberine Bridge Enzyme (BBE)	*P. somniferum*	Benzophenanthridine alkaloids	Constitutive antisense expression in cells	Changes in of benzophenatridine alkaloids content and major effects on cell growth and aminoacid synthesis	Park et al. 2002, 2003
	N-methyl-coclaurine 3'-hydroxylase (CYP80B1)	*P. somniferum*	Benzophenanthridine alkaloids	Constitutive over-/ antisense expression in cells	Reduced accumulation of benzophenatridine alkaloids and effects on cell growth and aminoacid synthesis	Park et al. 2002
Hyosciamus niger	Putrescine N Methyltransferase (PMT) + 6β hydroxylase (6H6)	*N. tabacum* Endogenous	Tropane alkaloids	Constitutive over-expression in hairy roots	Accumulation of high levels of scopolamine. Best results in trasformants with both PMT and 6H6 genes	Zhang et al. 2004
Artemisia annua	Farnesyl disphosphate synthase (FDS)	*G. arboreum*	Isoprenoid	Constitutive over-expression in hairy roots	Increased artemisinin content	Chen et al. 1999
				Constitutive over-expression in plant	Accumulation of artemisinin, higher in plant tissues than in roots	Chen et al. 2000
Duboisia myoporoides-D. leichhardti	Putrescine N Methyltransferase (PMT)	*N. tabacum*	Alkaloids	Constitutive over-expression in plant	Accumulation of N-methyl putrescine accumulation. No effect in tropane alkaloids content	Moyano et al. 2002

heterologous organisms, has yielded remarkable successes even for medicinal plants, especially for alkaloids and isoprenoids.

4.1.1 Engineering plant alkaloids

Alkaloids ascribed about 12,000 different nitrogenous compounds, found in about 20% of plant species, many of which are largely used as pharmaceuticals, such as the antitumoral vincristine and vinblastine, the narcotic morphine, the anti-cough depressant codeine, the oral antibacterial sanguinarine (Facchini 2001).

Several attempts have been reported on modifying expression of genes involved in the nicotine content in *Nicotiana tabacum*, which can be considered a model plant and a medicinal plant at the same time. Over-expression of tropinone reductase and hyoscyamine-6β-hydroxylase (*h6h*) has enhanced the synthesis of nicotine and related pyridin alkaloids (Rocha et al. 2002). As far as medicinal plants, the constitutive expression of tobacco *pmt* gene, encoding the putrescine N-methyltransferase, in *A. belladonna* plants and hairy roots did not significantly alter the alkaloid level (Sato et al. 2001), suggesting that the conversion of the putrescine and N-methylputrescine, the first two intermediate compounds in the synthesis of scopolamine from ornithine, are not rate-limiting for the synthesis of this compound. However, a 3-4-fold increase of scopolamine, a tropane alkaloid used as anticholinerginic sedative agent that acts on the parasympathetic nervous system, was achieved by over-expressing the *h6h* gene in *A. belladonna* (Yun et al. 1992). The H6H enzyme has a dual enzymatic activity since it catalyzes the hydroxylation of hyoscyamine to 6-β-hydroxyhyoscyamine and its further epoxidation to scopolamine (Facchini 2001).

Other alkaloids thoroughly studied for their pharmacological impact are those produced in *C. roseus*, the common Madagascar periwinkle. This plant species produces the monomeric alkaloids serpentine and ajmalicine, used as a tranquilizer and to reduce hypertension, respectively. Dimeric alkaloids from periwinkle, vincristine and vinblastine, and their semi-synthetic derivates, including vinorelbine and vinflunine, are used extensively in the treatment of many cancers. Dimeric alkaloids are synthesized at very low levels in the periwinkle and are restricted to specific leaf cell type (St Pierre et al. 1999). Synthesis of these bioactive secondary metabolites is quite complex since it involves two separate pathways, the terpenoid and the indole pathway, necessary for the synthesis of a converging intermediate, the strictosidine, and a total of over 20 different enzymes. Enzymatic studies coupled with expression analysis have demonstrated that activity of the tryptophan decarboxylase (*tdc*),

converting the tryptophan in tryptamine, coincides with alkaloid accumulation, while strictosidine synthase (STR) activity is relative stable (Meijer et al. 1993). Either *tdc* or *str* genes have been overexpressed in *C. roseus* cells or hairy roots in an attempt to enhance the level of terpenoid indole alkaloids (TIAs). Constitutive expression of *str* in cell culture proved to be useful to increase the alkaloid levels (Canel et al. 1998), while *tdc* overexpression triggered accumulation of the intermediate tryptamine, with no significant increase in TIAs (Goddijn et al. 1995). Alkaloid profiling has been also modified by over-expression of the *tdc* gene in other medicinal plants such as *Cinchona officinalis*, for quinine production (Geerlings et al. 1999) and *Peganum harmala,* producing psychotropic alkaloids (Berlin et al. 1993).

Another example of metabolic engineering of plant alkaloids is the modification of the synthesis of several important medicinal compounds belonging to the benzylisoquinoline alkaloid class, such as the antimicrobial berberine and sanguinarine, which has been attempted in *Coptis japonica* and the California poppy (*Eschscholzia californica* Cham). Constitutive expression of the scoulerine 9-*O*-methyltransferase (*smt*) gene in *C. japonica* cell cultures resulted in 20% higher SMT activity and a small increase in the accumulation of protoberberine alkaloids (Sato et al. 2001). Analogously, expression of *smt* gene in cultured California poppy cells led to a diversion of the metabolic flux towards the protoberberine alkaloid columbamine and away from benzophenenthridine (Sato et al. 2001).

Taken together these studies, have demonstrated that, more than increase the synthesis of a specific alkaloid, genetic modification of a single gene can divert this pathway, but much remains to be learnt before this class of bioactive compounds might be rationally engineered. As it will be discussed later in § 4.2.1, one possibility is to identify and over-express regulatory genes able to activate in a coordinate fashion the multiple genes involved in this complex pathway.

4.1.2 Engineering plant isoprenoids

Isoprenoids are the largest and most diverse family of natural products, with very diversified structures and chemical size. All the terpenoids have been long believed to be synthesized solely in the cytosol by condensation of units of isopentenyl diphosphate (IPP) and its allyl isomer dimethylallyl diphosphate (DMAPP), both originated from acetyl-CoA through the classical mevalonate (MVA) pathway, until the recent discover of an alternative plastidial pathway to IPP synthesis, starting from the 1-deoxy-D-xylulose phosphate (DXP). The cytosolic pathway supplies IPP for the

synthesis and subsequent precursor for the biosynthesis of sesquiterpenes and triterpenes, while monoterpenes, diterpenes and tetraterpenes are produced through the plastidial pathway (Mahmoud and Croteau 2002). This large family of compounds includes essential molecules, such as carotenoids, giberellins, abscisic acid, brassinosteroids, sterols and the phytol chains of chlorophyll, tocopherols and quinones. However, the majority are secondary metabolites of pharmacological interest, such as the volatile components of essential oils produced by the *Lamiaceae* species, and complex molecules like the anticancer drug taxol.

Monoterpenes comprise the major components of the essential oils of the *Lamiaceae* family, to which belong several medicinal and aromatic plants (sage, peppermint, basil to mention few of them). Biosynthesis of essential oils has been thoroughly studied in peppermint, which can be considered a model system for monoterpenes of the whole family. Synthesis of monoterpenes in mint is localized in glandular trichomes and originates in the leucoplasts of the secretory cells of these highly specialized structures. The flux through the monoterpene pathway has been modified in mint by over-expression of a gene encoding the deoxyxylulose phopshate reductoisomerase (DXR), which control the first committed step in the plastidial DXPS pathway (Mahmoud and Croteau 2001). Most transgenic plants accumulated more essential oils than control plants, with an increase up to 50%, without remarkable changes in the monoterpene composition compared to wild type plants. Furthermore, because the monoterpene synthase steps might be rate limiting (Wise and Croteau 1999), modified expression of these genes could increase yield and change composition of the monoterpenoid essential oil, as it has been demonstrated for ectopic expression of a 4S-limonene synthase in peppermint (*Mentha x piperita* L) and cornmint plants (*Mentha arvensis*) (Diemer et al. 2001; Krasnyanski et al. 1999).

A successful example of the metabolic engineering of cytosolic isoprenoids biosynthesis is the genetic modification of the artemisinin biosynthesis pathway. Artemisinin, extracted from the traditional Chinese herb *Artemisia annua* L, which has been used in China to treat fevers since A.D. 150, came into vogue as a modern malaria treatment after studies in Vietnam showed it reduced deaths from the illness by 97 percent. Most of the current pharmacological treatments against malaria are inefficient due to the selection of resistant forms of the parasite. The World Health Organization now recognizes that drugs combining antibiotics with artemisinin provide the most rapid defence against malaria and are the only ones to which the parasites has not developed resistance (WHO and UNICEF: Malaria World Report, 2005: http://rbm.who.int/wmr2005/-html/1-2.htm). Artemisinin is currently extracted from natural plants with a

very low content. Attempts to produce it by cell or organ tissue have been unsuccessful and chemical synthesis is not economically convenient (Abdin et al. 2003). Chemically, artemisinin is an endoperoxide sesquiterpenoid lactone (Klayman 1985), produced in the MVP pathway by two consecutive condensations of IPP with DMAPP by a farnesyl diphosphate synthase (FPPS), forming FPP, which is subsequently converted in artemisinin by a sesquiterpene cyclase (Bouwmeester 1999). Increased accumulation in artemisinin has been achieved by over-expressing the gene *fpps* in *A. annua* hairy roots and plants. In transgenic plants, the concentration of artemisinin was approximately 8-10 mg/g DW, about two- to three–fold higher than that in control plants, while in hairy roots the content of this sesquiterpene was 2-3 mg/g DW, which is three- to four-fold higher than in control roots (Chen et al. 1999; 2000). Optimization of enhanced production of this interesting plant secondary metabolites is, however, far to be reached, due to the involvement of a total of twelve genes, nine of which have already been cloned (Martin et al. 2003). The recent funding of research groups in USA by the Bill & Melinda Gates Foundation in 2004 may speed up the development of a genetically engineered form of artemisinin (Purcell 2006).

To the class of isoprenoids belongs also the tricyclic diterpene taxol (paclitaxel), initially isolated from the bark of *Taxus brevifolia*. Taxol is a potent antimitotic agent, acting by stabilizing microtubules and promoting microtubule assembly, rather than by depolymerizing microtubules, as found for vincristine and vinblastine. Since taxol is only available as a natural product, sources other than the bark of *T. brevifolia* have been investigated, including production of taxol from plant tissue culture. For a long period taxol was produced by chemical semi-synthesis from its precursor, 10-deacetylbaccatin III, extracted from leaves and twigs of the European yew, *T. baccata*, but presently Bristol-Meyer produced it solely from cell cultures of *T. chinensis*. Taxol is now used to treat ovarian, breast, and lung cancer and it has been prescribed to more than 1 million of patients. Despite the strong interest in this valuable natural anti-tumoral plant compound, successes in engineering its biosynthetic pathway are limited by the large number of enzymes involved (nineteen enzymatic steps from the universal diterpenoid progenitor GGPP) and lack of known regulatory genes. However, increased demands for taxol and its high cost have recently accelerated research towards the elucidation of its biosynthetic pathway, the responsible enzymes and underlying genes (Schoendorf et al. 2001; Jennewein et al. 2004), that may provide many promising targets for metabolic engineering of the production of taxol and its precursors.

4.2 Down-regulation of competitive or catabolic reactions

This approach deserves the same considerations that have been reported for over-expressing single rate-limiting genes. Though a down-regulation approach has been successfully applied in model and crop plants to remove undesirable plant compounds, in medicinal plants the main bottleneck for this technology is again the lack of thorough information on branching of complex biosynthetic pathways as well as on the endogenous plant catabolic degradation system of these compounds.

However, silencing of genes encoding key enzymes of relevant secondary metabolite pathways has been proved successful in some medicinal plants, either by antisense, co-suppression or RNAi technology. A significant decrease in the level of the undesirable menthofuran has been achieved in peppermint (*Mentha* x *piperita* L) through antisense suppression of the *mfs* gene, coding for the cytochrome P450 (+) menthofuran synthase (Mahmoud and Croteau 2001). Menthofuran reaches levels in environmentally stressed peppermint plants considered unacceptable by the pharmaceutical and industrial companies, because confer a bitter flavour to mint essential oils and promote off-color on storage. Accumulation of limonene in peppermint plants was achieved by co-suppression of the limonene-3-hydroxylase gene (Mahmoud and Croteau 2004). Down-regulation of the putrescine N-methyltransferase (PMT), the first committed step in both pyridine and tropane alkaloids, has been reported to reduce nicotine level in tobacco plants, with a concomitant unexpected increase in anatabine, whose synthesis does not require PMT (Chintapakorn and Hamill 2003). Antisense RNA-mediated suppression has been also reported for two genes involved in the biosynthesis of benzophenanthridine alkaloids in California poppy (Park et al. 2002), respectively the berberine bridge enzyme (BBE) and the N-methylcoclaurine 3'-hydroxylase (CYP80B1). These results have provided insight into the complex regulation of the benzylisoquinoline alkaloid biosynthesis, to which belong important pharmacological compounds, e.g. morphine, codeine, sanguinarine, rather than effectively increased specific alkaloids. BBE has been also knocked out by an antisense approach in opium poppy, resulting in an altered ratio of alkaloids in latex but not in roots (Frick et al. 2004).

RNAi technology has been also used recently in medicinal plants. A recent paper from Allen and coworkers (2004) reported the metabolic engineering of morphine biosynthesis in opium poppy (*Papaver somniferum* L.) by using RNAi to block the gene coding for the codeinone reductase (COR), the enzyme that catalyzes the reduction of codeinone to codeine and morphinone to morphine. Several COR genes are present in

the opium poppy, but the authors designed a hybrid RNAi construct to knock out all known members of this multigene family. Chemical analysis of the silenced transgenic lines showed that the amount of morphine was substantially lower and that, instead, higher level of (*S*)-reticuline, an intermediate compound located seven steps upstream the COR conversion in the morphine pathway. The authors speculated that this unexpected result might be due to feedback inhibition of the preceding enzymes or transporters, due to the resulting accumulation of codeinone and morphinone due to suppression of the COR or caused by other unpredictable transcriptional down-regulation of the up-stream enzymes. Alternatively, based on recent findings on the presence in plants of metabolon, multienzyme complex capable of metabolic channelling (Winkel 2004), a more fascinating explanation for this unexpected upstream control might be related to disruption of the morphinan metabolon caused by the silencing of COR, thus preventing the formation of its intermediates and resulting in the accumulation of (*S*)-reticuline. The relevance of metabolon formation in metabolic engineering of plant secondary metabolites will be discussed again when facing and solving possible pleiotropic effects associated to metabolic engineering (see § 6). Though not a medicinal plant *in sensu strictu*, in *Coffea canephora* RNAi has been used successfully to reduce the content of the alkaloid caffeine (Ogita et al. 2003).

As far as the feasibility of improving the synthesis of the end-product of interest through inhibiting eventual catabolic reactions, at our knowledge there are no examples in medicinal plants. Catabolism and degradation often occur simultaneously with synthesis, but, unfortunately, only the synthetic capacity has received sufficient attention in engineering secondary metabolites. Obviously, any strategy that is able to inhibit or avoid these degradative or catabolic processes may enhance total yield of a desired metabolite. However, catabolism of a secondary metabolite of interest may be avoided indirectly by promoting proper compartmentation through engineering genes involved in transport towards specialized cellular storage organelle (such as vincristine and vinblastine that are accumulated in the vacuole) or by a judicial choice of cell- tissue specific plant promoters, as it will be discussed in § 5.

4.3 Orchestrated regulation of sets of genes involved in the biosynthesis of plant secondary metabolites

Since it has been realized that the manipulation of single genes is of limited value in metabolic engineering, attention has shifted then towards

more complex and sophisticated strategies in which several steps in a given pathway are modified simultaneously to achieve optimal flux. Multipoint metabolic engineering is now beginning to replace single-point engineering as the best way to manipulate metabolic flux. This can be achieved either by over-expressing and/or suppressing several enzymes simultaneously or through the use of transcriptional regulators to control several endogenous genes.

4.3.1 Two or few are better than one: introducing multiple genes

Unlike bacteria, plants cannot normally co-express genes from polycistronic messengers. Introducing multiple genes into a target plant is quite challenging but not technically impossible, as elegantly proved for the increase in provitamin A in rice plants (Ye et al. 2000) or lignin pathway in forest tree (Li et al. 2003). Co-expression of multiple transgenes in a single tobacco plants was achieved by crossing independent transformed plants that expressed three different monoterpene synthases from lemon (Lucker et al. 2004), with an increase in endogenous terpenes as well as changes in the terpenoid profiles compared to untransformed wild-type plants. This is also the first report of transgenic plants expressing multiple foreign enzymes competing for the same substrate.

A nine-fold increase in the sedative compound scopolamine has been recently reported by simultaneously over-expressing the *pmt* and *h6h* genes, respectively encoding an up-stream and a down-stream rate-limiting enzyme in hairy roots of *Hyoscyamus niger* (Zhang et al. 2004). Compared to hairy roots over-expressing the single gene, the transgenic hairy roots expressing both *pmt* and *h6h* genes produced significantly higher levels of scopolamine, with the best line produced 411 mg/liter scopolamine, which was over nine times more than that the content of wild type roots (43 mg/liter). Transgenic hairy roots of *C. roseus* co-expressing a tryptophan decarboxylase (TDC) and a feed-back resistant anthranilate synthase subunit (ASα) have been produced (Hughes et al. 2004a). Interestingly, while the TDC line showed no significant increase in tryptamine level, hairy roots co-expressing the two genes synthesize much as six-fold amount of this crucial precursor in the synthesis of vincristine and vinblastine. This example offers another way of improved metabolic flux towards a desired end-product by mutagenesis of genes encoding enzymes subjected to a feed-back control. In fact, by over-expressing a mutated form of the AS α–subunit in periwinkle hairy roots, it has been proved that

this enzyme regulates the flux to tryptophan, a common precursor for alkaloid accumulation (Hughes et al. 2004b). Other possibilities of multiple gene co-expression have been attempted in plants other than medicinal ones, such as artificial gene-clusters introduced in plants using a vector system based on intron and intein-encoded endonuclease (Thomson et al. 2002) or the expression of polyproteins able to self-cleave, recently used to engineer ketocarotenoids in higher plants (Ralley et al. 2004). Finally, the success in plastidial transformation, although limited to a restricted number of plant species, opens also the possibility of introducing multiple genes assembled in polycistrons in the prokaryotic-like chloroplast genome, as it will be discussed in § 5.

4.3.2 Transcription factors

Although many successful results in increasing biosynthesis of plant secondary metabolites have been obtained by over-expression or down-regulation of single genes, in many cases, over-expression of an enzyme upstream in the pathway of the desired metabolite does not lead to increased production of that product, since secondary metabolites generally are not products of single genes, but are the results of multi-step, multi-enzymatic processes. Recent large-scale studies of gene expression coupled to metabolomic analyses have revealed that there is a coordinated metabolic regulation, mediated by regulatory genes that control the expression of the series of enzymes involved in a particular pathway. It has been demonstrated that the transcriptional control of the genes involved in metabolic pathways is the primary mechanism to regulate the final concentration of secondary metabolites in plants. Therefore, the identification of transcription factors (TFs) and relative genes able to simultaneously and coordinately controlling the transcription of multiple genes of a specific pathway is of great interest as biotechnological tool to enhance the production of plant bioactive molecules (for excellent reviews see Gantet and Memelink 2002; Broun 2004). The use of specific TFs would avoid the time-consuming steps of acquiring knowledge about all enzymatic steps of a poorly characterized biosynthetic pathway and, at the same time, allows the coordinated transcription of many genes belonging to a specific metabolic pathway.

The most well documented study of coordinated expression of biosynthetic genes in a medicinal plant driven by a TF is offered by the over-expression of ORCA3 in *C. roseus* cultured cells. The gene for ORCA3 (octadecanoid-responsive Catharanthus AP2-domain protein 2), a jasmonate-responsive gene, APETALA2 (AP2)-domain transcription factor from *C. roseus*, was isolated by T-DNA activation tagging (van der

Fit and Memelink 2000; 2001). *Orca3* over-expression in *C. roseus* cells resulted in enhanced expression of several metabolite biosynthetic genes and, consequently, in increased accumulation of terpenoid indole alkaloids (TIAs). Expression of TIA biosynthetic genes *tdc*, *str*, s*gd*, *cpr*, and *d4h* was increased in the *Orca3* over-expressing line, whereas *g10h* and *dat* genes were not induced, suggesting that they are not controlled by ORCA3. Genes encoding the α subunit of AS (AS α) and DXS, enzymes involved in primary metabolism leading to TIA precursor synthesis, were, instead, induced by *Orca3* over-expression. Transgenic cells that over-express ORCA3 accumulate significantly more tryptophan and tryptamine, and accumulate indole alkaloids when fed with the precursor secologanin (van der Fit and Memelink 2000). These data indicate that although ORCA3 is an important regulator of TIA biosynthesis, it is not sufficient to regulate the complete pathway.

Though not in medicinal plants, the feasibility of using regulatory genes for increasing the production of plant secondary metabolites other than alkaloids has been demonstrated in several other studies, as for phenylpropanoids by ectopic expression of plant *myb* and *bHLH* TF genes in *A thaliana*, *Petunia* or *N. tabacum* (Barkovich and Liao 2001; Schijlen et al. 2004). Studies in a number of systems suggest that many transcriptional regulators are capable of faithfully recognizing their homologous target genes in heterologous species, due to the conservation among the members of the same TF family. Therefore, it might be expected that over-expression in medicinal plants of TFs from other plants could be effective in modifying their metabolic profiles. To the MYB family belongs a novel TF, OsMYB4, isolated in rice, which has been recently described as able to increase cold and freezing tolerance when over-expressed in *Arabidopsis thaliana* plants (Vannini et al. 2004). Microarray analysis has revealed that *OsMYB4* over-expressing *Arabidopsis* plants express constitutively stress-responsive genes as well as genes involved in the metabolic pathway of secondary metabolites. Particularly, over-expression of *Osmyb4* gene in *Arabidospis* induces transcription of seven out of eight genes of the chorismate and aromatic amino acid biosynthesis, including also the chorismate mutase, the first enzyme responsible of the lateral chain leading to the synthesis of tyrosine and phenylalanine. Moreover *OsMYB4* controls the transcription of the genes for the phenylalanine ammonia-lyase (PAL), the cinnamate-4-hydroxylase (C4H) and the 4-coumarate:CoA ligase (4CL), involved in the phenylpropanoid biosynthesis (Immacolata Coraggio, personal communication). Over-expression of this transcription factor has increased the synthesis of chlorogenic acid in *Nicotiana tabacum* (Docimo et al. 2005) and of rosmarinic and salvianolic acid in *Salvia sclarea* (A. Leone

unpublished results), both compounds belonging to the class of phenylpropanoids.

Another large family of plant TFs is the WRKY proteins, which seems to exist exclusively in plants and low eukaryotes. The transcription of WRKY genes is strongly and rapidly upregulated in response to wounding, pathogen infection or abiotic stresses in numerous plant species (Eugelm et al. 2000). The WRKY family has 74 members in *Arabidopsis* (Ulker and Somssich 2004) and it would be interesting to exploit the role of the various members in triggering biosynthesis of bioactive secondary metabolites.

While the studies reported above have demonstrated that enhanced synthesis of plant secondary metabolites may be achieved through regulation of transcription of key biosynthetic genes or TFs, the formation of these plant compounds is a complex and dynamic process that involves multiple subcellular compartments, such as cytosol, endoplasmic reticulum, vacuoles and others, highlighting the central role of post-transcriptional regulation of secondary biosynthetic pathways. The relevance of these last aspects in successful metabolic engineering for plant molecules of therapeutic activity will be discussed in details in the next paragraph.

5. Improving transport and compartmentation

As discussed previously, in many species, the production of the astonishing number of secondary metabolites in plant cells relies on very well organized expression patterns of multiple genes. While gene expression is, in most cases, regulated at the transcriptional level and often influenced by the environment, the diversification of end-products, starting from few precursors and central intermediates, also depends on compartmentation of enzymes and reactions at the organ/tissue, cellular and subcellular level. Reasons for such tight developmentally related organization include the necessity to sequester toxic compounds in safe locales of the plant and the cell, the local availability of intermediates in some organs and tissues, the need to deliver end-products in plant parts where physiological and ecological interactions with other organisms, such as pollinators or pests, take place. Except for some notable cases, the lack of organization in undifferentiated cell cultures is often the cause of the failure of secondary metabolite production *in vitro* (De Luca and St Pierre 2000; Oksman-Caldentey and Inzé 2004). On the other hand, the comprehension, and possible manipulation, of cellular and molecular

mechanisms underlying secondary metabolite biosynthesis, transport and storage, is necessary for the adoption of successful metabolic engineering approaches and biotechnological applications.

By contrast with prokaryotic cells, plant cells present a number of compartments delimited by different kinds of membranes, that are responsible not only for marking the boundaries of production and/or storage sites, but also for intra- and intercellular transport.

It is now well known that isoprenoid production in plant cells is based on two parallel pathways leading to the same precursors but different end-products. Although some exchanges between the two pathways are possible, the acetate-mevalonate pathway, responsible for the production of isopenthenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) in the cytoplasm, leads to the synthesis of sesquiterpenes, triterpenes and other compounds, while, the GAP-pyruvate pathway determines the synthesis of IPP and DMAPP in plant plastids, where most hemi-, mono-, di- and tetraterpenes are produced and in various cases also accumulated (Croteau et al. 2000). Various highly specialized tissues and cells, such as glandular epidermis of flowers, resin blisters and ducts, glandular trichomes, secretory cavities and idioblasts, are involved in isoprenoid synthesis in higher plants, reflecting a complex pattern of organization at the tissue/cellular level at the basis of specialized functions and uses of end-products (McCaskill and Croteau 1997). The 3-hydroxy-3-methylglutaryl-coenzyme-A reductase (HMGR), a central enzyme in the acetate-mevalonate pathway, is present in different isoforms, encoded by small multigene families. Such isoforms show different tissue and developmental expression patterns and respond differently to environmental stimuli (McCaskill and Croteau 1998). Recent results in mint (Turner and Croteau 2004; Croteau et al. 2005) demonstrated that synthesis of menthol and other monoterpenes is not only compartimentalized at the tissue/cellular level, being confined to the glandular cap cells of secretory stage peltate glandular trichomes of the aerial part of the plant, but also at the subcellular level. In fact, different biosynthetic enzymes were immunolocalized in four cellular compartments, i.e. plastids (leucoplasts), endoplasmic reticulum, mitochondria, and cytoplasm (Fig. 16.2).

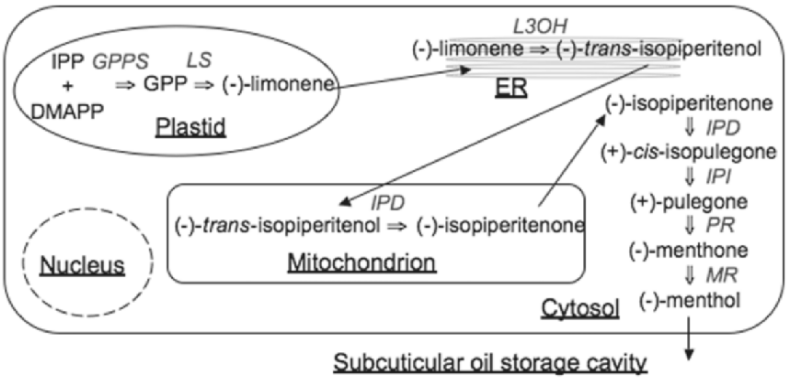


Fig. 16.2. Subcellular organization of (-)-menthol biosynthesis in the secretory gland cells of peppermint. Universal C_5 isoprenoid precursors IPP (isopenthenyl diphosphate) and DMAPP (dimethylallyl diphosphate) are produced in the plastid (leucoplast) through the GAP-pyruvate pathway. Subsequent enzymatic steps are localized in the plastid, the ER (endoplasmic reticulum), the mitochondrion, and the cytosol. Intermediates move between compartments and the end-product is secreted extracellularly in the subcuticular oil storage cavity. *GPPS* = geranyl diphosphate synthase; *LS* = (-)-limonene synthase; *L3OH* = (-)-limonene hydroxylase; *IPD* = (-)-*trans*-isopiperitenol dehydrogenase; *IPR* = (-)-isopiperitenone reductase; *IPI* = (+)-*cis*-isopulegone isomerase; *PR* = (+)-pulegone reductase; *MR* = (-)-menthone reductase. All enzymes are encoded by nuclear genes. Based on Croteau et al. (2005).

Results on enzyme localization were supported by those of transcripts and by the presence of specific signals in the correspondent gene sequences. Similarly, enzymes involved in subsequent steps of the biosynthesis of the diterpene taxol showed different subcellular localization (De Jong et al. 2006). A developmental regulated biosynthesis is also known for the tetraterpenes carotenoids, which is controlled by distinct regulatory mechanisms in chromoplasts of fruits and flowers and chloroplasts of green tissues (Hirschberg 2001).

Examples of various levels of organization patterns are also known for other secondary metabolite classes. The nicotine alkaloid in tobacco as well tropane alkaloids in other species are synthesized in roots and translocated to the aerial part for accumulation and storage (De Luca and St Pierre 2000; De Luca and Laflamme 2001; Yazaki 2006). Cells of different zones of the root, e.g. those of the pericycle and of the endodermis and outer cortex, harbour different enzymes and are specifically involved in different steps of tropane alkaloid biosynthesis in *A. belladonna* and other species (De Luca and St Pierre 2000; De Luca and Laflamme 2001). The first enzyme of the pathway leading to the synthesis of scopolamine (*S*-adenosyl-L-methionine-dependent putrescine-*N*-

methyltransferase) was localized in the perycicle (Suzuki et al.. 1999). Its presence in this tissue was linked to the unloading of the aminoacids ornithine and arginine, precursors of putrescine, from the vascular tissue into the pericycle (De Luca and St Pierre 2000).

The synthesis of the monoterpenoid indol-alkaloid vindoline (a precursor of the anticancer vinblastine) in *C. roseus* was stimulated by light and relied on the differential expression of several enzymes not only in various cell types, belonging to the internal phloem parenchyma of the young aerial organs, to the epidermis of developing leaves, and to leaf laticifers and idioblasts, but also in different subcellular compartments, such as the endoplasmic reticulum, the vacuole, the chloroplast, and the cytoplasm (McKnight et al. 1991; St Pierre and De Luca 1995; St Pierre et al. 1998; St Pierre et al. 1999; De Luca and St Pierre 2000; Irmler et al. 2000; Burlat et al. 2004; Kutchan 2005; Murata and De Luca 2005). Multiple cell types were also involved in the biosynthesis of various benzylisoquinoline-derived alkaloids in *P. somniferum* (opium poppy) (Weid et al. 2004; Kutchan 2005). For instance, some early enzymes for morphine biosynthesis were localized in phloem parenchyma cells of capsule and stem, whereas the late codeinone reductase in laticifers, where morphine accumulates. At the subcellular level, the biosynthesis of the lysine-derived quinolizidine alkaloids in lupins and other legumes occurs within the mesophyll chloroplasts of leaves, but further modifications, such as acylations, happen in the cytosol and the mitochondria, where the acyl donor is probably present. The final accumulation of end-products, however, is believed to occur in vacuoles of the epidermis cells of the leaf, where they can exert their defensive role (Suzuki et al. 1996; De Luca and St Pierre 2000). Indeed, the vacuole is the accumulation site of several toxic products, such as berberine in *C. japonica* and *Berberis wilsoniae,* nicotine in *Nicotiana spp.*, and others in various species (De Luca and St Pierre 2000; Hashimoto and Yamada 2003; Otani et al. 2005; Yazaki 2006).

An additional mechanism for "channeling" secondary metabolite production in plant cells was demonstrated with some phenylpropanoid biosynthetic enzymes (Kutchan 2005). Using advanced technologies, such as FRET (Fluorescence Resonance Energy Transfer), a non-invasive procedure for monitoring protein-protein interactions *in vivo*, and others, it was demonstrated the interaction and the (co)localization of two PAL isoforms with cytochrome P-450-dependent monoxigenase C4H, enzymes consecutively involved in the synthesis of coumaric acid starting from L-phenylalanine, in the endoplasmic reticulum and cytosol of tobacco cells (Achnine et al. 2004). Similarly, the interaction of chalcone synthase (CHS), chalcone isomerase (CHI) and dihydroflavonol 4-reductase (DFR)

was demonstrated not only *in vitro*, but also *in vivo,* particularly in specific subcellular compartments (*i.e.* the rough ER and vacuoles) of flavonoid synthesizing root cells (Burbulis and Winkel-Shirley 1999; Saslowsky and Winkel-Shirley 2001).

As discussed above, many secondary metabolite biosynthetic pathways show a cellular and/or a subcellular compartmentalization in different plant tissues and cell locales. This raises the question how intermediates and end-products move in a coordinated fashion from one tissue, cell, or subcellular compartment to another, and opens the way for additional engineering possibilities in transgenic cells.

An intercellular active transport mechanism based on specific ATP-binding cassettes (ABC) transporters was demonstrated in berberine producing *C. japonica* and *Thalictrum minus* cells (Yazaki et al. 2001; Sakai et al. 2002; Shitan et al. 2003; Terasaka et al. 2003a; Terasaka et al. 2003b). In *Berberis,* the terminal step of berberine biosynthesis occurs in vesicles that derive from ER and lately fuse with the central vacuole (Bock et al. 2002). Differences in transport mechanism with former species were attributed to the fact that while in *Berberis* berberine is produced and accumulates within the same cell, in *Coptis* and *Thalictrum* the production and accumulation sites are distinct (Yazaki 2005, 2006). In heterologous species, berberine can also be transported by a H^+-antiport mechanism (Yazaki 2005). An involvement of an ABC transporter has been identified in the leaves of *Nicotiana plumbaginifolia* in relation to the excretion of the diterpene sclareol onto the leaf surface (Jasinski et al. 2001), but it has been also hypothesized for nicotine in tobacco and for morphine intermediates in poppy (Yazaki 2005, 2006).

At the subcellular level, the vacuolar transport of berberine in *C. japonica* is dependent on a H^+-antiporter (Otani et al. 2005; Yazaki 2006). Similarly, it was suggested that a H^+-gradient-dependent transporter might have a role in vacuolar transport of anthocyanins in *A. thaliana* and tomato (Debeaujon et al. 2001; Mathews et al. 2003). On the other hand, an ABC transporter was found in maize tonoplast, being it required for anthocyanin accumulation in the vacuole (Goodman et al. 2004; Yazaki 2005). Interestingly, the same molecule, i.e. the barley flavonoid saponarin was imported in the vacuole via a H^+ antiporter in the homologous species, but through an ABC transporter in the heterologous species *Arabidopsis* (Frangne et al. 2002; Yazaki 2005). As previously mentioned, monoterpenoid production in mint proceeds from the plastids, to the endoplasmic reticulum, to the mitochondria, and finally to the cytoplasm, from where end-products are secreted to the subcuticolar cavity of the oil glands (Turner and Croteau 2004; Croteau et al. 2005). Simple diffusion of intermediates, somehow facilitated by their aqueous solubility and/or a

concentration gradient, seems the most likely mechanism in such intracellular transport, although some type of terpenoid carrier protein, a mitochondrial membrane pump, transient contacts between organelle membranes might help in some cases (Croteau et al. 2005). An ATP binding cassette transporter, however, has been hypothesized for active transport of end-products through the plasma membrane, because the final extracellular secretion is directional and selective for some monoterpene types.

Results discussed in this section not only indicate some interesting areas for future research, but also highlight some novel possibilities for metabolic engineering of secondary metabolite production in higher plants. The manipulation of genes encoding enzymes controlling critical biosynthetic steps is clearly important. However, based on recent results, it might be possible and probably necessary also to improve, through genetic engineering, the efficiency of long distance and cell to cell transport of metabolite intermediates and end-products, their extracellular secretion, and intracellular organelle to organelle movement (Hashimoto and Yamada 2003). The use of tissue specific promoters for developmentally regulated transgene expression, as well as the adoption of specific signals to address enzymes in the correct subcellular compartments are necessary in many cases.

Further, especially for the plastidial pathways, novel transformation procedures, such as the transformation of the plastome, might be used instead of the conventional nuclear transformation. In comparison with the latter, transgene expression in the plastidial genome shows some advantages, in this context mainly related to the expression level achievable and the possibility to express multiple genes in operons (Bock and Khan 2004). As far as the engineering of secondary metabolic pathways is concerned, plastidial transformation has been used so far in a few cases in tomato and tobacco: with bacterial, fungal and plant derived genes involved in carotenoid biosynthesis (Bock and Khan 2004), with the bacterial gene *ubiC* for p-hydroxybenzoic acid synthesis (Viitanen et al. 2004), and with a PAL (phenylalanine ammonia lyase) gene from *Arabidopsis* (C. Stettner, personal communication http://www.icongenetics.de). Other metabolic engineering applications were mainly related to the manipulation of the primary metabolism as well as to the production of recombinant proteins (Bock and Khan 2004).

6. Uncovering pleiotropic effects and avoiding failure in metabolic engineering of plant secondary metabolites

An increasing wealth of information is revealing that secondary metabolites are not solely involved in plant's defence mechanisms, but rather can also demarcate cellular and developmental differentiation. Therefore, to infer that secondary metabolites are not essential for the growth and development of a plant is an over-simplification that might underestimate eventual detrimental pleiotropic effects triggered by engineering secondary metabolites. Moreover, pleiotropic effects can rise because secondary metabolism forms a large interconnected network. Changes in the flux in one branch might lead to unexpected changes in other parts of the network.

Given the complexity and diversity of regulatory networks of secondary metabolism, it is impossible to make a generalization on how to introduce a novel high-flux pathway into a plant species without undesired effects. To avoid unexpected pleiotropic effects due to modifying one or few genes of a specific metabolic pathway, these interconnections have to be uncovered in advance, through accurate untargeted metabolic profiling beyond the strict boundaries of the pathway that is subjected to engineering (Trethewey 2004). Unexpected pleiotropic effects of modifying the expression of single or multiple genes in a plant are also revealed by microarray or proteomics analysis of the genetic modified plants. Integration of metabolomic and genomic tools have to be seen also as a tool to discover new key biosynthetic genes that can be engineered to enhance the production of bioactive natural compounds. Pleiotropic effects affecting plant growth and development may be less restrictive when engineering plant cells or hairy roots for scaling-up massive production of bioactive secondary metabolites by bio-fermentation.

Suggestions of how to avoid negative pleiotropic effects are offered by mechanisms by which plants naturally face this problem by transport and storage in a safe cell type (*e.g.* glandular cells of *Lamiacae* species) or other subcellular compartment (chloroplasts, vacuoles) or by secretion in the apoplast. For successful metabolic engineering of secondary metabolites in medicinal plants, tissue specificity and proper developmental regulation as well as proper subcellular localization of the desired product has to be taken into consideration. It follows that a judicial choice of the promoters, such cell or tissue-specific promoters, or contemporary over-expression of genes encoding intracellular transporters (as discussed in § 5) might be useful strategies.

Another way of optimising and increasing efficacy of metabolic engineering strategy comes from the recent discover that enzymes of complex metabolic pathways may be present in the cell in arrays of consecutive, physically associated enzymes assembled on membranes or other physical structures, to form multienzyme complexes, called metabolon (Winkel 2004; Jorgensen et al. 2005). Metabolon formation has to be seen as an evolutionary endogenous mechanism of plant cells to ensure efficient transformation of a common precursor into the end-product of a specific biosynthetic pathway by several different means. Firstly, catalytic efficiency is improved by bringing cooperating active enzymes into close proximity and, thereby, accelerating the time of synthesis of intermediates and reducing dilution of intermediates, which are both kinetic constraints of metabolite biosynthesis. At the same time, co-localization of multiple biosynthetic enzymes in a macromolecular complex secures a rapid conversion of potential labile/toxic intermediates into stable and not toxic molecules and, ultimately, prevents their secretion and probable degradation. Finally, metabolon formation might coordinate metabolic cross-talk by controlling either enzymes operating in different pathways or by a selection of intermediates shared between different metabolic pathways. It follows that gained knowledge on the molecular control of the metabolon formation and channeling of plant secondary metabolites will dramatically increase the potential of targeted metabolic engineering and enable the effective production of valuable phytopharmaceuticals.

7. Conclusions and perspectives

Despite the extensive work in the last few years, plant secondary metabolism remains still poorly characterized. Because metabolism is coordinated at many levels, the analysis of all regulatory levels is a prerequisite for comprehensive network analysis. Genetic maps of biosynthetic pathways are still far to be completed and very few regulatory genes of these pathways have been described and characterized, mainly in medicinal plants.

Technologies for gene discovery and plant transformation in medicinal plants are developing in advance to the understanding of the factors that control flux into specific routes of secondary metabolism. Knowledge of these factors is important to move from empirical to predictive metabolic engineering and is crucial to help bypass the low yield of various secondary metabolites in plants or cell cultures. However, it is becoming

increasingly clear that integrated analysis will be necessary in order to maximize understanding of metabolic networks. To date in plants this has only been attempted for few metabolic pathways or sub-networks. It is likely that the extension of such modular approaches will allow the identification of common regulatory motifs and enhance our understanding of metabolic regulation (Sweetlove and Fernie 2005).

As new genes for crucial enzymatic reaction are identified, proof-of concept may be obtained by preliminary testing the effect of their expression in *E. coli* (Willits et al. 2004) or other simple organisms. Another powerful tool towards predictive metabolic engineering is the rapid testing of pathway function of cloned genes, with unknown functions or for which kinetic parameters are unknown, by using functional chips, onto which mRNA-enzyme-fusion protein are immobilized (Jung and Stephanopoulos 2004).

Though integrative analysis in plants, based on correlations between genes, proteins and metabolites, is at the beginning, it remains likely that further advances in the development of network biology in the near future will give a tremendous impulse to successful application of metabolic engineering in medicinal and crop plants.

Acknowledgements

We apologize for omitting other interesting contributions to advancements in metabolic engineering of medicinal and crop plants, that have not be cited for lack of space or simply for our ignorance.

This work was partially supported by the Italian Research Program of National Interest (PRIN 2005) by Ministry of Education, University and Research (MIUR).

Contribution number 75 from the CNR-IGV, Institute of Plant Genetics, Portici (Naples), Italy

References

Abdin MZ, Israr M, Rehman RU, Jain SK (2003) Artemisinin, a novel antimalarial drug: biochemical and molecular approaches for enhanced production. Planta Med 69: 289-299

Achnine L, Blancaflor EB, Rasmussen S, Dixon RA (2004) Colocalization of L-phenylalanine ammonia-Lyase and cinnamate 4-hydroxylase for metabolic channeling in phenylpropanoid biosynthesis. Plant Cell 16: 3098-3109

Allen RS, Milligate AG, Chitty IA, Thisleton J, Miller JAC, Fist AJ, Gerlach WL, Larkin PJ (2004) RNAi-mediated replacement of morphine with the non-narcotic alkaloid reticuline in oppium poppy. Nat Biotechnology 22: 1559-1566

Ayora-Talavera T, Chappell J, Lozoya-Gloria E, Loyola-Vargas VM (2002) Overexpression in *Catharanthus roseus* hairy roots of a truncated hamster 3-hydroxy-3methylglutaryl-CoA reductase gene. Appl Biochem Biotechnol 97: 135-145

Bajaj YPS (1999) Transgenic Medicinal Plants. Series: Biotechnology in Agriculture and Forestry, vol 45 Springer Verlag, Berlin, Heidelberg, New York

Barkovich R, Liao JC (2001) Metabolic Engineering of Isoprenoids. Metab Eng 1: 27-39

Berlin J, Rugenhagen C, Dietze P, Fecker LF, Goddijin OJM, Hoge JHC (1993) Increased production of serotonin by suspension and root cultures of *Peganum harmala* transformed with a tryptophan decarboxylase cDNA from *Catharanthus roseus*. Transgenic Res 2: 336-344

Bock A, Wanner G, Zenk M (2002) Immunocytological localization of two enzymes involved in berberine biosynthesis. Planta 216: 57-63

Bock R, Khan MS (2004) Taming plastids for a green future. Trends Biotech 22: 311-318

Bouwmeester HJ, Wallaart TE, Janssen MH, van Loo B, Jansen BJ, Posthumus MA, Schmidt CO, De Kraker JW, Konig WA, Franssen MC (1999) Amorpha-4,11-diene synthase catalyses the first probable step in artemisinin biosynthesis. Phytochemistry 52: 843-854

Brandle J, Richman A, Swanson AK, Chapman BP (2002) Leaf ESTs from *Stevia rebaudiana*: a resource for gene discovery in diterpene synthesis. Plant Mol Biol 50: 613-622

Briskin DP (2000) Medicinal plants and phytomedicines. Linking plant biochemistry and physiology to human health. Plant Physiol 124: 507-514

Broun P (2004) Transcription factors as tools for metabolic engineering in plants. Curr Opin Plant Biol 7: 202-209

Burbulis IE, Winkel-Shirley B (1999) Interactions among enzymes of the *Arabidopsis* flavonoid biosynthetic pathway. In: Proc Natl Acad Sci USA 96, 12929-12934

Burlat V, Oudin A, Courtois M, Rideau M, St Pierre B (2004) Co-expression of three MEP pathway genes and geraniol 10-hydroxylase in internal phloem parenchyma of *Catharanthus roseus* implicates multicellular translocation of intermediates during the biosynthesis of monoterpene indole alkaloids and isoprenoid-derived primary metabolites. Plant J 38: 131-141

Canel C, Lopes-Cardoso MI, Whitmer S, Van der Fits L, Pasquali G, Van der Heijden R, Harry J, Hoge C, Verpoorte R (1998) Effects of overexpression of strictosidine synthase and tryptophan decarboxylase on alkaloid production by cell cultures of *Catharanthus roseus*. Planta 205: 414-419

Chen DH, Liu CJ, Ye HC, Li GF, Liu BY, Meng YL, Chen XY (1999) Ri-mediated transformation of *Artemisia annua* with a recombinant farnesyl

diphosphate synthase gene for artemisinin production. Plant Cell Tiss Org Cult 57: 157–162

Chen DH, Ye HC, Li GF (2000) Expression of a chimeric farnesyl diphosphate synthase gene in *Artemisia annua* L. transgenic plants via *Agrobacterium tumefaciens*-mediated transformation. Plant Sci 155: 179-185

Chintapakorn Y, Hamill JD (2003) Antisense-mediated down-regulation of putrescine N-methyl transferase activity in transgenic *Nicotiana tabacum* L. can lead to elevated levels of anabatine at the expense of nicotine. Plant Mol Biol 53: 87-105

Croteau R, Kutchan TM, Lewis NG (2000) Natural products (secondary metabolites). In: Buchanan B, Gruissem W and Jones R (eds) Biochemistry & Molecular Biology of Plants, American Society of Plant Physiologists, Rockville, Maryland, pp 1250-1318

Croteau R, Davis E, Ringer K, Wildung M (2005) (-)-Menthol biosynthesis and molecular genetics. Naturwissenschaften 92: 562-577

De Luca V, St Pierre B (2000) The cell and developmental biology of alkaloid biosynthesis. Trends Plant Sci 5: 168-173

De Luca V, Laflamme P (2001) The expanding universe of alkaloid biosynthesis. Curr Opin Plant Biol 4: 225-233

Debeaujon I, Peeters AJM, Leon-Kloosterziel KM, Koornneef M (2001) The TRANSPARENT TESTA12 gene of *Arabidopsis* encodes a multidrug secondary transporter-like protein required for flavonoid sequestration in vacuoles of the seed coat endothelium. Plant Cell 13: 853-872

De Jong JM, Liu Y, Bollon AP, Long RM, Jennewein S, Williams D, Croteau RB (2006) Genetic engineering of taxol biosynthetic genes in *Saccharomyces cerevisiae*. Biotechnol and Bioengin 93: 212-224

Diemer F, Caissard JC, Moja S, Chalchat JC, Jullien F (2004) Altered monoterpene composition in transgenic mint following the introduction of 4S-limonene synthase. Plant Physiol Biochem 39: 603-614

Docimo T, Sacco A, Dalpiaz F, De Tommasi N, Coraggio I (2005) Boosting the phenylpropanoid pathway in *Nicotiana tabacum* plants by over-expression of the *Osmyb4* transcription factor. In: Proceedings of the XLIX Italian Society of Agricultural Genetics Annual Congress, Potenza, Italy, Poster Abstract - A.01 ISBN 88-900622-6-6

Edwards R (2004) No remedy in sight for herbal ransack. New Sci 181: 10-11

Eulgem T, Rushton PJ, Robatzek S, Somssich IE (2000) The WRKY superfamily of plant transcription factors. Trends Plant Sci 5: 199-206

Facchini PJ (2001) Alkaloid biosynthesis in plants: biochemistry, cell biology, molecular regulation and metabolic engineering applications. Annu Rev Plant Physiol Plant Mol Biol 51: 29-61

Frangne N, Eggmann T, Koblischke C, Weissenbock G, Martinoia E, Klein M (2002) Flavone glucoside uptake into barley mesophyll and *Arabidopsis* cell culture vacuoles. Energization occurs by H^+-antiport and ATP-Binding Cassette-type mechanisms. Plant Physiol 128:726-733

Frick S, Chitty JA, Kramell R, Schmidt J, Allen RS, Larkin PJ, Kutchan TM (2004) Transformation of opium poppy (*Papaver somniferum* L.) with

antisense berberine bridge enzyme gene (anti-bbe) via somatic embryogenesis results in an altered ratio of alkaloids in latex but not in roots. Transgenic Res 13: 607-613

Gang DR, Wang J, Dudareva N, Nam KH, Simon JE, Lewinsohn E, Pichersky E (2001) An investigation of the storage and Biosynthesis of Phenylpropenes in Sweet Basil. Plant Physiol 125: 539-555

Gantet P, Memelink J (2002) Transcription factors: tools to engineer the production of pharmacologically active plant metabolites. Trends in Pharm Sci 12: 563-569

Geerlings A, Hallard D, Martinez-Caballero A, Lopes-Cardoso I, Van der Heijden R, Verpoorte R (1999) Alkaloid production by a *Cinchona officinalis* 'Ledgeriana" hairy root culture containing constitutive expression constructs of tryptophan decarboxylase and strictosidine synthase cDNAs from *Catharanthus roseus*. Plant Cell Rep 19: 191-196

Glase V (1999) Billion dollar market blossoms as botanicals take root. Nat Biotechnol 17:17-18

Goddijn OJ, Pennings EJ, van der Helm P, Schilperoort RA, Verpoorte R, Hoge JH (1995) Overexpression of a tryptophan decarboxylase cDNA in *Catharanthus roseus* crown gall calluses results in increased tryptamine levels but not in increased terpenoid alkaloid production. Transgenic Res 4: 315-323

Goodman CD, Casati P, Walbot V (2004) A multidrug resistance-associated protein involved in anthocyanin transport in *Zea mays*. Plant Cell 16: 1812-1826

Goossens A, Hakkinen ST, Laasko I, Seppanen-Laakso T, Biondi S, De Sutter V, Lammertyn F, Nuutila AM, Zabeau M, Inzé D, Oksman-Caldentey K-M (2003) A functional genomics approach toward the understanding of secondary metabolism in plant cells. Proc Natl Acad Sci US 100: 8595-8600

Hashimoto T, Yamada Y (2003) New genes in alkaloid metabolism and transport. Curr Opin Biotech 14:163-168

Hirschberg J (2001) Carotenoid biosynthesis in flowering plants. Curr Opin Plant Biol 4:210-218

Hong SB, Peebles CA, Shanks JV, San KY, Gibson SI (2006) Expression of the Arabidopsis feedback-insensitive anthranilate synthase holoenzyme and tryptophan decarboxylase genes in *Catharanthus roseus* hairy roots. J Biotechnol 122: 28-38

Hughes EH, Hong S-B, Gibson SI, Sha nks JV, San K-Y (2004a) Metabolic engineering of the indole pathway in *Catharanthus roseus* hary roots and increased accumulation of tryptamine and serpentine. Metab Eng 6: 268-276

Hughes EH, Hong S-B, Gison SI, Shanks JV, San K-Y (2004b) Expression of a feed-back resistant anthranilate synthase in *Catharanthus roseus* hairy roots provides evidence for tight regulation of terpenoid indole alkaloid level. Biotechnol Bioeng 86: 718-27

Irmler S, Schroder G, St-Pierre B, Crouch NP, Hotze M, Schmidt J, Strack D, Matern U, Schroder J (2000) Indole alkaloid biosynthesis in *Catharanthus roseus*: new enzyme activities and identification of cytochrome P450 CYP72A1 as secologanin synthase. Plant J 24:797-804

Jasinski M, Stukkens Y, Degand H, Purnelle B, Marchand-Brynaert J, Boutry M (2001) A plant plasma membrane ATP Binding Cassette-type transporter is involved in antifungal terpenoid secretion. Plant Cell 13:1095-1107

Jennewein S, Wildung MR, Chau M, Walker K, Croteau R (2004) Random sequencing of an induced *Taxu*s cells cDNA library for identification of clones involved in Taxol biosynthesis. Proc National Aca Sci USA, 101: 9149-9154

Jorgensen K, Rasmussen AV, Morant M, Nielsen AH, Bjarnholt N, Zagrobelny M, Bak S, Moler BL (2005) Metabolon formation and metabolic channeling in the biosinthesis of plant natural products. Curr Opin Plant Biol 8: 280-291

Joshi K, Chavan P, Warudel D, Patwardnan B (2004) Molecular markers in herbal drug technology. Current Science 2: 159-165

Jung GY, Stephanopoulos G (2004) A functional protein chip for pathway optimization and in vitro metabolic engineering. Science 304: 428-431

Kaufman PB, Cseke LJ, Warber S, Duke JA, Brielmann HL (1999) Natural Products from Plants. CRC Press, Boca Raton, USA

Klayman DL (1985) Qinghasosu (artemisinin): An antimalarial drug from China. Science 228: 1049-1055

Krasnyanski S, May RS, Loskutov A, Ball TM, Sink KC (1999) Transformation of the limonene synthase gene into peppermint (*Mentha piperita* L.) and the preliminary studies on the essential oil profiles of single transgenic plants. Theor Appl Genet 99: 676-682

Kutchan TM (2005) A role for intra- and intercellular translocation in natural product biosynthesis. Curr Opin Plant Biol 8: 292-300

Li L, Zhou Y, Cheng X, Sun J, Marita JM, Ralph J, Chiang VL (2003) Combinatorial modification of multiple lignin traits in trees through multigene cotransformation. Proc Natl Acad Sci USA, 100: 4939-4944

Lucker J, Schwab W, van Hautum B, Blaas J, van der Plas LHW, Bouwmeester HJ, Verhoeven HA (2004) Increased and altered fragrance of tobacco plants after metabolic engineering using three monoterpene synthases from lemon. Plant Physiol 134: 510-519

Mahmoud SS, Croteau RB (2001) Metabolic engineering of essential oil yield composition in mint by altering expression of deoxyxylulose phosphate reductoisomerase and menthofuran synthase. Proc Natl Acad Sci USA 98: 8915-8920

Mahmoud SS, Croteau R (2002) Strategies for transgenic manipulation of monoterpene biosynthesis in plants. Trends Plant Sci 7: 366-373.

Mahmoud SS, Williams M, Croteau R (2004) Cosuppression of limonene-3-hydroxylase in peppermint promotes accumulation of limonene in the essential oil. Phytochemistry 65: 547-554

Martin VJJ, Pitera DJ, Withers ST, Newman DJ, Jay D Keasling JD (2003) Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. Nat Biotech 21: 796-802

Mathews H, Clendennen SK, Caldwell CG, Liu XL, Connors K, Matheis N, Schuster DK, Menasco DJ, Wagoner W, Lightner J, Wagner DR (2003) Activation tagging in tomato identifies a transcriptional regulator of

anthocyanin biosynthesis, modification, and transport. Plant Cell 15: 1689-1703

McCaskill D, Croteau R (1997) Prospects for the bioengineering of isoprenoid biosynthesis. Adv Biochem Engin/Biotech 55: 107-146

McCaskill D, Croteau R (1998) Some caveats for bioengineering terpenoid metabolism in plants. Trends Biotech 16: 349-355

McKnight TD, Bergey DR, Burnett RJ, Nessler CL (1991) Expression of enzymatically active and correctly targeted strictosidine synthase in transgenic tobacco plants. Planta 185: 148-152

Meijer AH, Verpoorte R, Hoge JHC (1993) Regulation of enzymes and genes involved in terpenoid indole alkaloid biosynthesis in *Catharanthus roseus*. J Plant Res 3: 145-164

Moyano E, Fornale S, Palazon J, Cusido RM, Bagni N, Pinol MT (2002) Alkaloid production in *Duboisia* hybrid hairy root cultures overexpressing the *pmt* gene. Phytochemistry 59: 697-702

Murata J, De Luca V (2005) Localization of tabersonine 16-hydroxylase and 16-OH tabersonine-16-O-methyltransferase to leaf epidermal cells defines them as a major site of precursor biosynthesis in the vindoline pathway in *Catharanthus roseus*. Plant J 44: 581-594

Noel JP, Austin MB, Bomati EK (2005) Structure-function relationship in plant phenylpropanoid biosynthesis. Curr Opin Plant Biol 8: 249-253

Ogita S, Uefuji H, Yamaguchi Y, Koizumi N, Sano H (2003) Production of decaffeinated coffee plants by genetic engineering. Nature 423: 823

Oksman-Caldentey K-M, Inzé D (2004) Plant cell factories in the post-genomic era: new ways to produce designer secondary metabolites. Trends Plant Sci 9: 433-440

Otani M, Shitan N, Sakai K, Martinoia E, Sato F, Yazaki K (2005) Characterization of vacuolar transport of the endogenous alkaloid berberine in *Coptis japonica*. Plant Physiol 138: 1939-1946

Park SU, Yu M, Facchini PJ (2002) Antisense RNA-mediated suppression of benzophenanthridine alkaloid biosynthesis in transgenic cell cultures of California poppy. Plant Physiol 128: 696-706

Park SU, Yu M, Facchini PJ (2003) Modulation of berberine bridge enzyme levels in transgenic root cultures of California poppy alters the accumulation of benzophenanthridine alkaloids. Plant Mol Biol 51: 153-164

Pichersky E, Gang DE (2000) Genetics and bioschemistry of secondary metabolites in plants: an evolutionary perspective. Trends Plant Sci 5: 439-445

Pickersky E, Noel JP, Dudareva N (2006) Biosynthesis of plant volatiles: Nature's Diversity and Ingenuity. Science 211: 808-811

Purcell K (2006) Gates Foundation invests $ 42.6 Million in Malaria Drug Research. HerbalGram 69: 24-252

Ralley L, Enfissi EMA, Misawa N, Schuch W, Branley PM, Fraser PD (2004) Metabolic engineering of ketocarotenoids formation in higher plants. Plant J 39: 477-86

Richter U, Rothe G, Fabian AK, Rahfeld B, Drager B (2005) Overexpression of tropinone reductases alters alkaloid composition in *Atropa belladonna* root cultures. J Exp Bot 56: 645-652

Rischer H, Oresic M, Seppanen-Laakso T, Katajamaa M, Lammertyn F, Ardiles-Diaz W, Van Montagu MC, Inze D, Oksman-Caldentey KM, Goossens A (2006) Gene-to-metabolite networks for terpenoid indole alkaloid biosynthesis in *Catharanthus roseus* cells. Proc Natl Acad Sci USA 2006 (in press)

Rocha P, Stenzel O, Parr A, Walton N, Christou P, Drager B, Leech MJ (2002) Functional expression of tropinone reductase I (*trf*) and hyoscyamine-6b-hydroxylase (*h6h*) from *Hyoscyamus niger* in *Nicotiana tabacum*. Plant Sci 162: 905-913

Rothe G, Hachiya A, Yamada Y, Hashimoto T, Drager B (2003) Alkaloids in plants and root cultures of *Atropa belladonna* overexpressing putrescine N-methyltransferase. J Exp Bot 54: 2065-2070

Sakai K, Shitan N, Sato F, Ueda K, Yazaki K (2002) Characterization of berberine transport into *Coptis japonica* cells and the involvement of ABC protein. J Exp Bot 53: 1879-1886

Saslowsky D, Winkel-Shirley B (2001) Localization of flavonoid enzymes in *Arabidopsis* roots. Plant J 27: 37-48

Sato F, Hashimoto T, Hackiya A, Tamura K, Chioi KB, Morishige T, Fujimoto H, Yamada Y (2001) Metabolic engineering of plant alkaloid biosyntheis. Proc Natl Acad Sci USA 98: 367-372

Scheindlin S (2005) Antimalarials: shortage and searches. Mol Interv 5(4): 201-206

Schoendorf A, Rithner CD, Williams RM, Croteau R (2001) Molecular cloning of a cytochrome P450 taxane 10ß -hydroxylase cDNA from *Taxus* and functional expression in yeast. Proc Natl Acad Sci USA 98: 1501-1506.

Schijlen EGWM, de Vos RCH, van Tunen AJ, Bovy AG (2004) Modification of flavonoid biosynthesis in crop plants. Phytochemistry 65: 2631-2648

Shitan N, Bazin I, Dan K, Obata K, Kigawa K, Ueda K, Sato F, Forestier C, Yazaki K (2003) Involvement of CjMDR1, a plant multidrug-resistance-type ATP-binding cassette protein, in alkaloid transport in *Coptis japonica*. Proc Natl Acad Sci USA 100: 751-756

St Pierre B, De Luca V (1995) A cytochrome P-450 monooxygenase catalyzes the first step in the conversion of tabersonine to vindoline in *Catharanthus roseus*. Plant Physiol 109: 131-139

St Pierre B, Laflamme P, Alarco A-M, De Luca V (1998) The terminal O acetyltransferase involved in vindoline biosynthesis defines a new class of proteins responsible for coenzyme A dependent acyl transfer. Plant J 14: 703-713

St Pierre B, Vazquez-Flota FA, De Luca V (1999) Multicellular compartmentation of *Catharanthus roseus* alkaloid biosynthesis predicts intercellular translocation of a pathway intermediate. Plant Cell 11: 887-900

Suzuki H, Koike Y, Murakoshi I, Saito K (1996) Subcellular localization of acyltransferases for quinolizidine alkaloid biosynthesis in *Lupinus*. Phytochemistry 42:1557-1562

Suzuki K, Yamada Y, Hashimoto T (1999) Expression of *Atropa belladonna* putrescine N-methyltransferase gene in root pericycle. Plant Cell Physiol 40: 289-297

Sweetlove LJ, Fernie AR (2005) Regulation of metabolic networks: understanding metabolic complexity in the systems biology. New Phytol 1: 9-24

Terasaka K, Sakai K, Sato F, Yamamoto H, Yazaki K (2003a) *Thalictrum minus* cell cultures and ABC-like transporter. Phytochemistry 62: 483-489

Terasaka K, Shitan N, Sato F, Maniwa F, Ueda K, Yazaki K (2003b) Application of vanadate-induced nucleotide trapping to plant cells for detection of ABC proteins. Plant Cell Physiol 44: 198-200

Thomson JM, Lafayette PR, Schmidt MA Parrott WA (2002) Artificial gene-cluster engineered into plants using a vector system based on intron – and intein-encoded endonucleases. In Vitro Cell Dev Biol Plant 38: 537-542

Trethewey RN (2004) Metabolic profiling as an aid to metabolic engineering in plants. Curr Opin Plant Biol 7: 196-201

Turner GW, Croteau R (2004) Organization of monoterpene biosynthesis in *Mentha*. Immunocytochemical localizations of geranyl Diphosphate Synthase, Limonene-6-Hydroxylase, Isopiperitenol Dehydrogenase, and Pulegone Reductase. Plant Physiol 136: 4215-4227

Ulker B, Somssich IE (2004) WRKY transcription factors: from DNA binding towards biological function. Curr Opin Plant Biol 7: 491- 498

Unterlinner B, Lenz R, Kutchan TM (1999) Molecular cloning and functional expression of codeinone reductase: the penultimate enzyme in morphine biosynthesis in the opium poppy *Papaver somniferum*. Plant J 18: 465- 475

Van der Fits L and Memelink J (2000) ORCA3, a Jasmonate-Responsive Transcriptional Regulator of Plant Primary and Secondary Metabolism. Science 289: 205-297

van der Fits L, Hilliou F, Memelink J (2001) T-DNA activation tagging as a tool to isolate regulators of a metabolic pathway from a genetically non-tractable plant species. Transgenic Res 10: 513-521

Vannini C, Locatelli F, Bracale M, Magnani E, Marsoni M, Osnato M, Mattana M, Baldoni E, Coraggio I (2004) Overexpression of the rice Osmyb4 gene increases chilling and freezing tolerance of *Arabidopsis thaliana* plants. Plant J 37: 115-127

Viitanen PV, Devine AL, Khan MS, Deuel DL, Van Dyk DE, Daniell H (2004) Metabolic engineering of the chloroplast genome using the *Escherichia coli ubiC* gene reveals that chorismate is a readily abundant plant precursor for p-hydroxybenzoic acid biosynthesis. Plant Physiol 136: 4048-4060

Vines G (2004) Herbal harvests with a future: towards sustainable sources for medicinal plants, Plantlife International, www.plantlife.org.uk

Weid M, Ziegler J, Kutchan TM (2004) From the cover: The roles of latex and the vascular bundle in morphine biosynthesis in the opium poppy, *Papaver somniferum*. Proc Natl Acad Sci USA 101: 13957-13962

Willits MG, Giovanni M, Prata RTN, Kramer CM, De Luca V, Steffens JC, Graser G (2004) Biofermentation of modified flavonoids: an example of in vivo diversification of secondary metabolites. Phytochemistry 65: 31-41

Wink M (1999). Functions of plant secondary metabolites and their exploitation in biotechnology. Ann Plant Reviews, 3, CRC Press, Boca Raton, USA, pp 370

Wink M, Schimmer O (1999) Modes of actions of defensive secondary metabolites. In: Wink M (ed) Functions of Plant Secondary Metabolites and their exploitation in Biotechnology. CRC Press, Boca Raton, FL, pp 17-112

Winkel BS (2004) Metabolic channeling in plants. Annu Rev Plant Biol 55: 85-107

Wise ML, Croteau R (1999) Biosythesis of monoterpenes. In: Cane DE (ed) Comprehensive Natural Products Chemistry, vol 2, Isoprenoids Including Carotenoids and Steroids. Elsevier, Oxford, pp 97–153

Yazaki K, Shitan N, Takamatsu H, Ueda K, Sato F (2001) A novel *Coptis japonica* multidrug-resistant protein preferentially expressed in the alkaloid-accumulating rhizome. J Exp Bot 52: 877-879

Yazaki K (2005) Transporters of secondary metabolites. Curr Op Plant Biol 8: 301-307

Yazaki K (2006) ABC transporters involved in the transport of plant secondary metabolites. FEBS Lett 580: 1183-1191

Ye X, Al-Babili S, Kloti A, Zhang J, Lucca P, Beyer P, Potrykus I (2000) Engineering the provitamin A (β-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. Science 287: 303-305

Yun D-J, Hashimoto T, Yamada Y (1992) Metabolic engineering of medicinal plants: transgenic *Atropa belladonna* with an improved alkaloid composition. Proc Natl Acad Sci USA 89: 11799-11803

Zhang L, Ding R, Chai Y, Bonfill M, Moyano E, Oksman-Caldentey KM, Xu T, Pi Y, Wang Z, Zhang H, Kai G, Liao Z, Sun X, Tang K (2004) Engineering tropane biosynthetic pathway in *Hyoscyamus niger* hairy root cultures. Proc Natl Acad Sci USA 101: 6786-6791

17

Potential market for bio-based products in the context of European greenhouse gas reduction strategies

Bengt Hillring, Matti Parikka

Swedish University of Agricultural Sciences (SLU), Department of Bioenergy, P.O. Box 7061, 750 07 Uppsala, Sweden (e-mail: Bengt.Hillring@bioenergi.slu.se)

1. Introduction

In the European Union, it is estimated that 50% of the primary energy supplied by different renewable energy sectors comes from wood (EurObserver 2003). The wood energy sector is based on sustainable use of European forests and to a certain extent recovered wood, forest industrial by-products (e.g. bark and sawdust) and wood from plantations: energy from agriculture, i.e. agricultural residues, e.g. straw, also contributes to the EU energy supply (European Commission 1997).

According to the White Paper for renewable sources *COM(97) 599* (European Commission 1997), the European Union is required to double the amount of renewable energy in the European primary energy supply from 6% in 1997 to 12% by 2010. The majority of the additional renewable energy needed to achieve the set target could come from biomass, which means that, additionally, 26 Mtoe (1 EJ) of biomass could be used for energy (Parikka 2005a). A possible alternative for covering future demand for renewable energy is increased utilisation of wood from plantations, energy crops, recovered wood, forest and agricultural residues and by-products from the wood-processing industry. At present, the utilisation

P. Ranalli (ed.), Improvement of Crop Plants for Industrial End Uses, 509–521.

of these materials, mainly forest residues, is low compared with the estimated potential (Parikka 2005a & 2005b).

The future development of renewable sources of energy within the EU also depends on the environmental impacts and different technical and non-technical barriers (Madlener et al 2005; Parikka 2005a).

One important aspect in the appraisal of these resources is the assessment of potential, which forms a part in the setting of targets and limits for practical utilisation (Parikka 2002 & 2004). Another important aspect is public opinion – how to improve the acceptability of renewable resources. There are several other factors to take into account, e.g. noise, visual intrusion, environmental concerns and even other non-technical obstacles (Madlener et al 2005).

In the Kyoto process, the European Union declared the intention to reduce greenhouse gas emissions (CO_2, CH_4, N_2O etc.) by 8% by the year 2010, with reference to the 1990 emission levels (European Commission 2006). Biomass, and especially woody biomass, could substitute fossil fuels, e.g. in electricity and heat supply; thereby, contributing substantially to the reduction of greenhouse gas emissions (GHG), principally CO_2 from fossil fuel combustion (Karlopoulos and Tsikardini 2004).

The fast growing market for wood fuel and other renewables in Europe in recent years has already raised questions concerning their influence on the environment. Some of these resources are already physically available in the form of recovered wood, residues from agriculture and forestry, as well as from related industries (Parikka 2005a). Established over the past 10 years, the bioenergy trade is now a viable alternative for countries and regions lacking resources, and a business opportunity for countries with an oversupply (Alakangas et al 2002).

2. Existing European strategies for green house gas reduction

The following short summary presents the existing European strategies for green house gas reduction.

Table 17.1. Existing European strategies for green house gas reduction strategies.

Renewable energy in the EU	
88/349/	Council Recommendation of 9 June 1988 on developing the exploitation of renewable energy sources in the Community.
COM(97) 599	Communication from the Commission - Energy for the future: renewable sources of energy - White Paper for a Community strategy and action plan.
COM(2000) 769	Green Paper - Towards a European strategy for the security of energy supply.
Directive 2001/77/	Promotion of electricity produced from renewable energy sources in the internal electricity market.
Directive 2002/91/	Energy performance in buildings.
Directive 2003/30/	Promotion of the use of biofuels or other renewable fuels for transport.
Directive 2003/96/	Directive on energy taxation.
Directive 2004/8/	Promotion on co-generation (combined heat and power).
SEC(2004) 547	Communication from the Commission to the Council and the European parliament: The share of renewable energy in the EU. A Commission Report in accordance with the Article 3 of Directive 2001/77/EC proposals for concrete actions.
COM (2004) 366	A Commission staff working document: The share of renewable energy in the EU country profiles. An overview of renewable energy sources in the enlarged European Union.
European Commission Memo (2004)	Renewable energy to take off in Europe ? - 2004 - Overview and scenario for the future.
EU climate strategy and emission rights trading	
ECCP – programme I	The first European Climate Change Programme (ECCP) was launched by the Commission in 2000.
ECCP – programme II	The second European Climate Change Programme (ECCP) was launched by the Commission in 2005.
COM(2000) 87	Green Paper on greenhouse gas emissions trading within the European Union.
Directive 2003/87	Emissions trading - National allocation plans.
Kyoto Agreement	The EU and its Member States ratified the Kyoto Protocol in late May 2002.

Source: Eurolex (2005); European Commission (2006).

3. Use of biomass fuels in Europe

In the late 80's, few statistics on renewable energy, either at the EU level or in the member states, existed. The Council recommendation of 1988 *88/349/EEC* (Eurostat 2004) stipulated that the member states, in collaboration with the Eurostat should establish a statistical system for Renewable Energy Sources (RES) (Eurostat 2006).

The use of renewable energy sources in the EU (25) are presented in Table 17.2. The total gross inland energy consumption in 2003 was 72 124 PJ in 2003. The share of all renewables was 6%, about 4 329 PJ, and the share of biomass and biofuels was 3,9%, about 2 880 PJ (Eurostat 2006).

Table 17.2. Gross inland energy consumption in the EU(25).

All categories	PJ	%
- Natural gas	17 086	23,0
- Oil	27 038	38,0
- Coal	13 163	18,0
- Nuclear	10 517	15,0
- Renewables	4 229	6,0
Total	72 134	100,0
Renewables		
- Wind	159	3,7
- Solar	26	0,6
- Hydro	1 045	24,1
- Geothermal	221	5,1
- Biomass and biofuels	2 880	66,5
Total	4 229	100,0

Source: (Eurostat 2006)

The share of wood in the EU (25) energy supply (wood as fuel in households and wood as fuel in industry and power plants) is about 50% of all renewables, about 2 000 PJ (Thrän and Kaltschmitt 2002).

4. Potential of biomass fuels in Europe

The total biomass energy potential in Europe is about 9 EJ/a (Kaltschmitt 1999; Kaltschmitt and Neubarth 2000; Thrän and Kaltschmitt 2002). The total practically available annual biomass quantity in Europe is approximately 5.2 EJ (Table 17.3): this figure is based on statistics from 20 European countries (Vesterinen and Alakangas 2001). The share of available woody biomass for fuel is about 4 EJ per year (e.g. forest industry by-products, forest and logging residues, firewood, recovered wood, etc.). A comparison between the available potential with the current use on European level, indicates approximately 32% of existing biomass energy potential is used (Kaltschmitt 1999; Thrän and Kaltschmitt 2002; Eurostat 2006).

Table 17.3. Biomass for energy resources in Europe, PJ per year.

PJ/a	Forest residues	Solid ind. by-products	Ind. black liquors	Firewood	Wood wastes	Densified wood fuels	Other biomass fuels	Peat	Total
Austria	150. 0	50.0	0.0	40.0	18.0	3.0	9.0	0.0	270.0
Belgium	7.0	13.0	8.0	0.0	3.0	0.0	0.0	0.0	31.0
Denmark	11.0	5.0	0.0	3.0	0.0	4.0	46.0	0.0	69.0
Finland	96.0	47.0	135.0	49.0	0.0	1.0	11.0	165.0	504.0
France	38.0	42.0	0.0	258.0	111.0	0.3	412.0	0.0	861.3
Germany	142.0	40.0	0.0	0.0	81.0	0.0	511.0	0.0	774.0
Greece	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ireland	3.0	7.0	0.0	3.0	1.0	0.0	0.0	40.0	54.0
Italy	0.0	36.0	0.0	83.0	24.0	0.0	0.0	0.0	143.0
Netherlands	4.0	3.0	0.0	0.0	45.0	1.0	24.0	0.0	77.0
Portugal	68.0	27.0	22.0	0.0	0.0	0.0	0.0	0.0	117.0
Spain	59.0	87.0	23.0	12.0	52.0	0.0	386.0	0.0	619.0
[1]Sweden	238.0	46.4	125.0	27.0	27.0	18.0	22.0	13.0	516.4
UK	16.0	12.0	0.0	27.0	175.0	0.0	70.0	0.0	300.0
Estonia	30.0	0.0	0.0	0.0	0.0	0.0	19.2	30.0	79.2
Latvia	8.0	12.0	0.0	32.0	0.0	1.0	0.0	15.0	68.0
Poland	101.0	68.0	16.0	26.0	40.0	0.0	205.0	122.0	578.0
Romania	0.0	23.0	3.0	58.0	0.4	0.0	0.1	0.0	84.5
Slovakia	6.0	0.1	5.0	3.0	3.0	0.1	13.0	0.0	30.2
Slovenia	2.0	7.0	0.0	8.0	0.1	0.0	0.0	0.0	17.1
Total	979	526	337	629	581	28	1 728	385	5 193

Source:Vesterinen and Alakangas (2001).

[1] The figure for Sweden has been updated. The new total figure for Sweden is 516 PJ per year (Lönner et al 1998; Parikka 2003).

The wood energy potential is especially important in countries where forests cover a considerable part of the total land area, e.g. the Nordic countries (Europe). By-products from forest industry, such as bark and sawdust in sawmills and bark in pulp mills, are currently the largest commercially used biomass source (Kaltschmitt and Neubart 2000; Parikka 2002 & 2004).

The types of the more important wood energy resources differ. In Finland and Sweden it is either wood or wood residues harvested during or after harvesting of industrial round wood or by-products from forest industry; whereas, e.g. in Germany approximately two-thirds of the estimated resources is straw. These differences are partly caused by the different means of estimating the resources, but the main reason is the land use (Vesterinen and Alakangas 2001).

The group "Other biomass resources", which includes biomass types such as short rotation coppice (e.g. Willow), energy grass (e.g. Reed canary grass) and straw, is especially dominant in France, Germany, Spain and Poland. In these countries, the other biomass resources means mainly straw (Vesterinen and Alakangas 2001).

Availability of wood residues (wood residues including bark, sawdust, wood chips etc.) at European level varies rather little and the quantity is dependant on industrial use of round wood. A major part of wood residues are used for industrial purposes: bark is used for energy purposes and an increasing quantity of sawdust is used for production of densified biofuels, i.e. pellets and briquettes, raw wood chips are mostly used for pulp (Parikka 2002; FAO 2004a). Statistics, e.g. from FAO (2004a) indicate increased production of split firewood at European level.

The industrial round wood sector is particularly important, typically having a large impact on wood fuel prices (as does pulp and paper, and some other industries). Round wood (i.e. timber, veneer, pulp wood, etc.) is used as industrial raw material and as construction material. However, of the total round wood quantity about 40% of the total quantity is primary or secondary process residues, suitable only for energy production, e.g. for the production of densified biofuels (FAO 2004b).

5. Potential market for biomass fuels in Europe

5.1 Markets for biomass in Europe

Biomass is typically a crop grown and used in the local society. Bulky material and high transport costs have kept it local up to modern time.

Trade between countries and regions is an effective way to distribute specialities among companies and organisations in a more specialised world. Trade usually increase rationalisation in the forest sector and liberalisation strongly supports this development. Problem could be that areas like the forestry sector internationally is not very well developed to meet this movement and discussions are going on a global scale how to meet this (Hillring 2006).

International statistics is not covering this trade in biofuel completely. Efforts to summarise global trade and the trade patterns have been done by different authors (NUTEK 1993; Hillring and Trossero 2006).

Within the European union, trade of industrial biofuels occurred some decade ago: early trade paths of olive stones from southern (Spain) and recovered wood from central Europe (Germany) were to Swedish district heating plants (Alakangas et al 2002). Later, trade from countries in the Baltic area increased (Latvia, Estonia Poland, Russia) both for regular round wood destined for the forest products industry in the Nordic countries and for low quality timber and residues for energy companies in Sweden, Denmark, Germany and the Netherlands.

The driving force behind the biofuel trade in Europe is based on efforts to reach the limitations in emissions dictated by the Kyoto protocol and other international agreements. Higher international energy prices are another important factor. The situation differs among the regions in Europe. Some areas such as the Nordic countries are rich in forest resources and have a large demand for biofuels. Other countries such as Russia have even greater forest resources but virtually no demand for biofuels. In central Europe, some countries are rich in forests (such as France) but biomass from the agricultural sector dominates. The most extreme example is the Netherlands, with a dense population and an extensive agriculture sector, the majority of the biofuel supply needs to be imported, even though it is expected to grow in the future.

5.2 European trade patterns

Solid biofuels such as wood residues, pellets and wood chips are traded within Europe and reached a level of almost 50 PJ per year in 1999

(Vesterinen and Alakangas 2000). Biofuel trade between European countries is a growing interest, because international trade can provide fuels at lower prices. In several cases, the national biomass market is underdeveloped for organised international trade; however, projects may benefit countries with unexploited biomass resources where fuels are available on an international market. Although there may be (even notable) cross-border trade of e.g. domestic firewood between neighbouring countries, this trade is more or less occasional and beyond official statistics (AFB-Net 2000). In some countries (e.g. Portugal), the statistics reveal traded biomass amounts, but as the source/destination countries are unknown, these flows cannot be included in the total figure. However, in some cases the trading countries are known, but the traded biomass types are not (AFB-Net 2000).

The largest volumes of biofuel are traded from the Baltic countries (Estonia, Latvia, and Lithuania) to the Nordic countries (especially Sweden and Denmark, but also Finland). Some volumes are also traded from Finland to other Nordic countries, and between neighbouring countries in Central Europe, especially the Netherlands, Germany, Austria, Slovenia and Italy. The traded biofuels include densified wood fuels (pellets and briquettes) and industrial by-products (sawdust, wood chips), and recovered wood in Central Europe. The annual production of wood pellets in Europe is estimated to be about 12-13 million tonnes. There is also intercontinental trade of biofuels. Sweden imports biofuels from Canada, and Italy imports firewood from Northern Africa. In addition, Germany exports firewood to the Middle and Far East (AFB-Net 2000).

Scandinavian biofuel markets have increased and national energy policies have contributed strongly to this trend (Vesterinen and Alakangas 2000). Taxes on energy with a clear environmental profile were introduced during the early 1990s in Scandinavian countries. Fossil fuels for heat production are heavily taxed, whereas biofuels are untaxed. In electricity production, all fuels are untaxed, and consumers pay the tax. In Finland and Sweden, investment support promoted growth capacities and contributed to biofuel demand.

A new project, EUBIONET II (2006) is underway, and covers the European trade of biofuels. No results are currently available yet, but preliminary studies from Sweden indicate that trade has doubled compared with 5-6 years ago. There has been a change in the mix of biofuels imported to Sweden, with a significant increase in wood pellets, tall oil and ethanol.

5.3 Fuel prices

Energy prices in general have increased over the past 30 years, especially during the past 3 to 4 years, which also provides more incentive for alternative energy sources such as wood for energy (Figure 17.1). Oil prices increased substantially in 2004-2005 due to several factors such as increased global demand, limited capacity for oil refineries, political violence in key production areas e.g. the Middle East, and natural catastrophes e.g. in the Southern United States. Levels over 60 USD per barrel were reached at some periods. This development on the oil market forced prices on all energy to increase, which created new energy solutions and provided an opportunity for alternatives such as wood fuel to be competitive (NUTEK 1993).

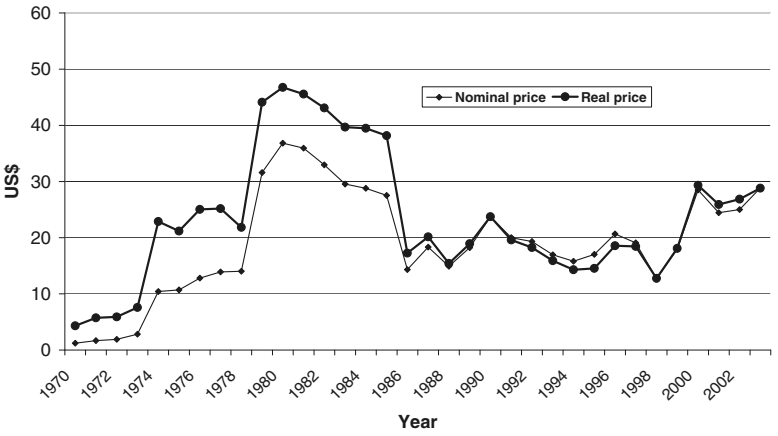


Fig. 17.1. Nominal and real prices of light crude oil, 1970-2004, Source: STEM (2005b) Periodical.

Table 17.4 provides examples of European wood fuels prices, which are the result of increased trade on wood fuels between European countries and which have led to well established international wood fuels prices (Hillring 2006). There is of course a range of prices set by the market situation, production cost and cost of competing fuels. By-products traded at 3-4 €/GJ, wood chips around 3.5-4.5 €/GJ and wood pellets around 5-6 €/GJ. Swedish market prices (2005) for wood fuels to heating plants vary between 3.4 €/GJ for by-products and 6.2 €/GJ for wood pellets (Hillring 2006). A survey

of the prices of different fuels was undertaken in 1999 (Vesterinen and Alakangas 2000; AFB-Net 2000). Since then, oil prices have risen significantly, which has also influenced wood fuel prices.

Table 17.4. Minimum, maximum and average fuel prices (including taxes) in the 18 selected European countries (1999).

Fuel	Country Minimum, €/GJ		Country Maximum, €/GJ		Average €/GJ
Forest residues	Germany	1.02	Italy	8.33	3.42
By-products, forest products industry	Romania	0.58	Poland	9.07	2.38
Firewood	Slovakia	1.01	UK	14.00	5.26
Wood waste	Ireland	-4.00	Poland	3.31	0.97
Refined wood fuels	Latvia	3.24	Germany	18.22	8.37
Other biomass	Slovakia	0.83	Poland	12.00	4.68
Peat	Finland& Latvia	2.10	Ireland	3.75	2.83
Heavy fuel oil	Slovakia	1.40	Ireland	12.00	6.74
Light Fuel oil	Slovakia	3.10	Denmark	14.30	6.74
Natural gas	Slovakia	1.10	Italy	16.21	5.80
Coal	Poland	1.19	Germany	12.78	4.53

Source: Vesterinen and Alakangas (2000); AFB-Net (2000).

6. Conclusion and discussion

Biomass has the potential to substitute for the use of fossil fuels in Europe; however, the exploitation of this potential is only advisable if there are promising economic and/or environmental effects.

A transition process is now taking place and many conditions are changing, including policy level decision-making, forestry practices, agricultural policy and the forest industry. In addition, the international biofuel trade will be an important factor in the future: the global benefit of displacing fossil fuel with modern biofuel use is obvious, as life cycle analyses indicate that biofuel supply systems are neutral in terms of emissions CO_2 to the atmosphere.

According to the White Paper, the overall aim is to double the share of renewable energy from 6% to 12% of the total energy consumption in the EU by 2010: a major part of this renewable energy could come from biomass. This means that an additional 1 EJ of biomass per year could be used for energy in the European Union. A possible alternative is to cover the future demand for renewable energy by increased utilisation of wood from plantations, energy crops, forest and agricultural residues, and by-products from the wood-processing industry.

In the Kyoto summit, the European Union pledged its intention to reduce greenhouse gas emissions (CO_2, CH_4, N_2O etc) by 8% by the year 2010, compared to the 1990 emission levels. Biomass, and especially woody biomass, could be used to substitute fossil fuels, e.g., in electricity generation and heat supply. Biomass will therefore substantially contribute to the reduction of Greenhouse Gas (GHG) emissions, mainly CO_2, from fossil fuel combustion.

Practical experience and research has shown that locally sustainable biofuel systems can be operated across Europe. All impacts are manageable with straightforward measures that are generally an extension of, e.g. good conventional forestry and agricultural practice. Another way of doing this is to issue recommendations for the removal of residues, e.g. logging residues, with the purpose of minimizing the risks for long-term adverse effects on soil fertility and the environment.

The biofuel trade is a viable alternative for countries without their own resources and for countries with an oversupply. The international biofuel market prices are established in Europe, however volumes are still limited. In the future, both the use and the trade of biofuels is expected to increase, in Europe, the self supply, high fossil energy and electricity prices and international environmental agreements are the main driving forces for the development of this industry.

The share of wood in the EU(25) energy supply (wood as fuel in households and wood as fuel in industry and power plants) is about 50% of all renewables, about 2,0 EJ. This means that the share of wood in the EU(25) energy system is about 2.8% The corresponding share of biomass and biofuels is about 3.9% and about 6.0%for all renewables.

It was found that the potential of different biomass fuels in Europe is about 9 EJ per year (the current use is about 2,9 EJ per year), meaning that about 32% of the total resources are currently in use. The annual biomass fuel quantity practically available in Europe is about 5.2 EJ per year.

References

AFB-Net (2000) Export-import possibilities and fuel prices – Task 2. AFB-Net V – Targeted actions in bioenergy network – Part 1. Country reports. VTT Energy, National coordinators and CEE subcontractors. Jyväskylä, Finland

Alakangas E, Hillring B, Nikolaisen LS (2002) Trade of Solid Biofuels and Fuel Prices in Europe. In: proceedings 12th European Biomass Conference – Biomass for Energy

EUBIONETT (2006) http://www.eubionet.net

EurObserver (2003) http://www.caddet.org

Eurolex (2005) 88/349/EEC. Council Recommendation of 9 June 1988 on developing the exploitation of renewable energy sources in the Community. http://europa.eu.int/eur-lex/en/index.html

European Commission (1997) COM(97) 599. Communication from the Commission - Energy for the future. Renewable sources of energy - White Paper for a Community strategy and action plan. November 1997. http://europa.eu-int/comm/off/white/index_en.htm

European Commission (2006). EU climate strategy and emission rights trading . http://europa.eu.int/comm/environment/climat/eccp.htm.

Eurostat (2004) Database. Renewable energy sources statistics in the EU, Iceland and Norway. Data 1989-2000. KS-46-02-080-EN-C, http://epp.eurostat.cec.eu.int.

Eurostat (2006) Database. Energy statistics http://epp.eurostat.cec.eu.int

FAO (2004a) FAOSTAT database. Forestry data. http://www.fao.org

FAO (2004b) Forestry Department. Trade and sustainable forest management – Impacts and interactions. Analytic study of the global project GCP/INT/775/JPN. Impact assessment of forest products trade in the promotion of sustainable forest management

Hillring B (2006) World trade in forest products and wood fuel. In print. Accepted for publication in Biomass and Bioenergy

Hillring B, Trossero M (2006) International wood fuel trade - an overview . Energy for Sustainable Development. Vol X, No 1. pp 33-41

Kaltschmitt M (1999) Utilization of biomass in the German energy sector. In: Hake J-F, Bansal N and Kleemann M (eds) Strategies and technologies for greenhouse gas mitigation. Ashgate, Aldershot

Kaltschmitt M and Neubarth J (2000) Biomass for energy – An option for covering the energy demand and contributing to the reduction of GHG emissions. In workshop proceedings: Integrating biomass energy with agriculture, forestry and climate change policies in Europe. December 2000. Imperial College, London

Karlopoulos E, Tsikardini E (2004) Life Cycle Analysis of Energy Wood Production. ECHAINE, "Energy Wood Production Chains in Europe". ENK5-CT2002-00623. Deliverable 29

Lönner G, Danielsson B, Vikinge B, Parikka M, Hektor B, Nilsson P (1998) Cost and supply of wood fuels at an intermediate time range. Swedish University of Agricultural Sciences. Department of Forest-Industry-Market Studies. Report No 51. Uppsala. In Swedish, English summary

Madlener R, Kaufman M, van Hezik C (2004) Market and policy overview and promising energy wood production chains in selected European countries. ECHAINE, "Energy Wood Production Chains in Europe". ENK5-CT-2002-00623. Deliverable 21

NUTEK (1993) Forecast for Biofuel Trade in Europe - The Swedish Market in 2000. Swedish National Board for Industrial and Technical Development. B 1993:10, Stockholm

Parikka M (2002) Global Biomass Fuel Resources. In: proceedings 1st World Conference on Pellets. Stockholm, pp 191-197

Parikka M. (2003) Swedish Wood Fuel Supply. In: proceedings Renewable Energy 2003 - Modern situation, problems and trends. Peterhof Congress Centre, St Petersburg, Russia, pp 134-139

Parikka M (2004) Global Biomass Fuel Resources. Biomass and Bioenergy 27: 613-620

Parikka M (2005a) Potential of Energy Wood in Europe. ECHAINE "Energy Wood Production Chains in Europe" ENK5-CT-2002-00623. Deliverable D26 & D27

Parikka M (eds) (2005b) Energy Wood Production Chains in Europe. ECHAINE, "Energy Wood Production Chains in Europe". ENK5-CT-2002-00623. Brochure

STEM (2005a) Energy in Sweden. Facts and Figures 2005. Swedish National Energy Administration. Eskilstuna, Sweden, Also available on: http://www.stem.se in Swedish and in English

STEM (2005b) Periodical. Prices for biofuels, peat etc. Swedish National Energy Administration. Eskilstuna, Sweden, Also available on: http://www.stem.se in Swedish

Thrän D and Kaltschmitt M (2002) Biomass for a Sustainable Energy Provision Systems – State of Technology, Potentials and Environmental Aspects. In: Sayigh A (ed) Workshop proceedings. World Renewable Energy Congress. Cologne

Vesterinen P, Alakangas E (2000) Export-import possibilities and fuel prices in 20 European countries. Task 2. AFB-net. VTT Energy. Jyväskylä, Finland

Vesterinen P, Alakangas E (2001) AFB-NET. Part 1. Final Report. VTT Energy. Jyväskylä, Finland

Index