



## PROJECT FINAL REPORT

**Grant Agreement number:** 227420

**Project acronym:** COMOFARM

**Project title:** Contained Molecular Farming – Controllable Contained Systems for High Yield and Consistency

**Funding Scheme:** Small collaborative project

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# **1 FINAL PUBLISHABLE SUMMARY**

## **1.1 Executive summary**

The CoMoFarm project was conceived to facilitate the development of high-yielding production systems based on plants, plant tissues and plant cells for the large-scale production of pharmaceutical and industrial proteins. The concept behind the project was to develop ways to standardize the growth and behavior of plants (and plant tissues and cells) so that pharmaceutical proteins can be produced to a consistent yield and quality.

The project focused on the comparative analysis of several plant species and four different plant-based systems: hydroponic plants, root cultures, cultivated moss and suspension cells. The best systems were developed further by optimizing the physical, chemical and biological parameters of cultivation to improve yields and product quality. As part of this process, we also developed automated systems to monitor plants and maintain them in peak health, ultimately leading to a full production platform including downstream processing, taking into consideration the current regulatory environment for plant-derived pharmaceutical proteins.

The CoMoFarm project was the first to look at plant-based production systems holistically, with a view to optimizing the entire production train from cell to pure protein, specifically focusing on pharmaceutical industry standards. The project introduced innovative concepts such as in-process monitoring and the automation of environmental parameter control to optimize product yield, quality and homogeneity. We also developed novel downstream processing technologies and customized bioreactors and hydroponic environments tailored for plants producing pharmaceutical proteins.

Ultimately, the results from the CoMoFarm project will help to reduce the costs involved in the production of pharmaceutical and industrial proteins and ensure that pharmaceuticals from plants are produced to the highest possible regulatory standards.

## **1.2 A summary description of project context and objectives**

The aims of the CoMoFarm project were to develop high-yielding production systems based on plants, plant tissues and plant cells for the large-scale production of pharmaceutical and industrial proteins. The concept behind the project was to develop ways to standardize the growth and behavior of plants (and plant tissues and cells) and thus achieve consistent yields and product quality.

The project involved the comparative analysis of four different plant-based systems (hydroponic plants, root cultures, cultivated moss and suspension cells) and several different plant species. The best performers were used for strain and process optimization. Automated systems were developed for plant monitoring and maintenance, ultimately leading to a full production platform including downstream processing. Platform and process development were carried out with due consideration for the regulatory landscape relevant to plant-derived pharmaceutical products.

The CoMoFarm project was the first to look at plant-based production systems holistically, with a view to optimizing the entire production train from cell to pure protein, specifically focusing on pharmaceutical industry standards. The project involved many innovative elements including online in-process monitoring and the automated control of environmental parameters to optimize product yield, quality and homogeneity. Novel downstream processing technologies and enhanced bioreactor and hydroponic facility designs were developed to keep plants and plant cells in peak health.

Ultimately, the results from the CoMoFarm project will help to reduce the costs involved in the production of pharmaceutical and industrial proteins and to ensure that pharmaceuticals from plants are produced to the highest possible standards.

The specific objectives of the project:

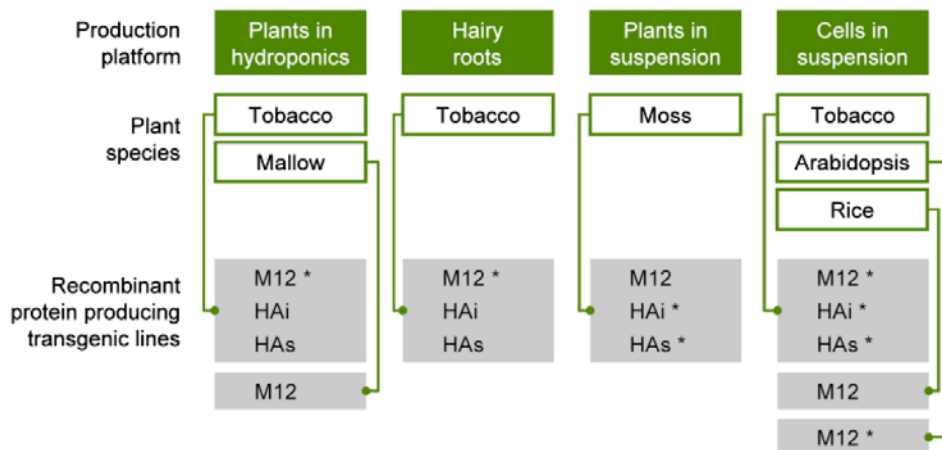
- Evaluate four types of contained systems based on plants, hairy roots, moss and plant cell suspension cultures
- Evaluate different species to determine which is the most suitable for the production of three candidate pharmaceutical proteins
- Optimize nutritional and physical parameters to maximize the production of pharmaceutical target proteins in containment
- Optimize protein stability and secretion
- Develop non-invasive monitoring systems
- Develop automated systems for plant, hairy root and plant suspension cell cultivation
- Achieve proof-of-concept for at least one plant-based production platform including comprehensive process evaluation according to good manufacturing practices (GMP).

### **1.3 A description of the main scientific and technical achievements**

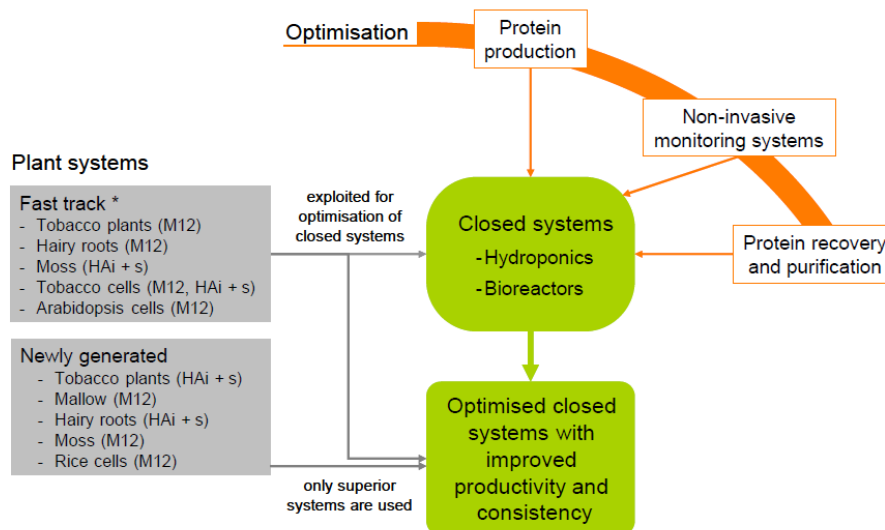
The CoMoFarm project was divided into six research work packages as listed below, plus a seventh work package for project management:

- WP1, Comparison of different plant production platforms
- WP2, Optimization of protein production, stability and secretion
- WP3, Development of automated contained systems
- WP4, Protein recovery and purification
- WP5, Protein characterization
- WP6, Proof-of-concept and process evaluation
- WP7, Project management

The first 18 months of the project focused on the first three work packages. The overall aim of WP1 was to establish the different production platforms (i.e. transgenic plants, hairy roots, moss cultures and plant suspension cells) producing three candidate pharmaceutical molecules: the human antibody M12, and secreted and membrane-integrated variants of influenza virus hemagglutinin (HA), which we named HAs and HAI (**Figure 1**). Some platform/product combinations were available prior to the launch of the project, allowing their immediate optimization in WP2 (**Figure 2**). The first task in WP1 was to complete the matrix of platform/product combinations so all three candidate products were expressed in all available platforms. This work was largely complete by the end of the first year, although there was a minor delay with the moss bioreactor system reflecting initial cloning problems. The second task in WP1 was to achieve small-scale cultivation with each system, providing material for the third and most extensive task, which was the extensive characterization of each platform and the corresponding products to enable the first milestone to be achieved, i.e. the selection of the most suitable platform for further development.



**Figure 1** Host/target matrix showing the three target proteins and the different host systems. Combinations indented for fast-track development (available at the start of the project) are identified with an asterisk.



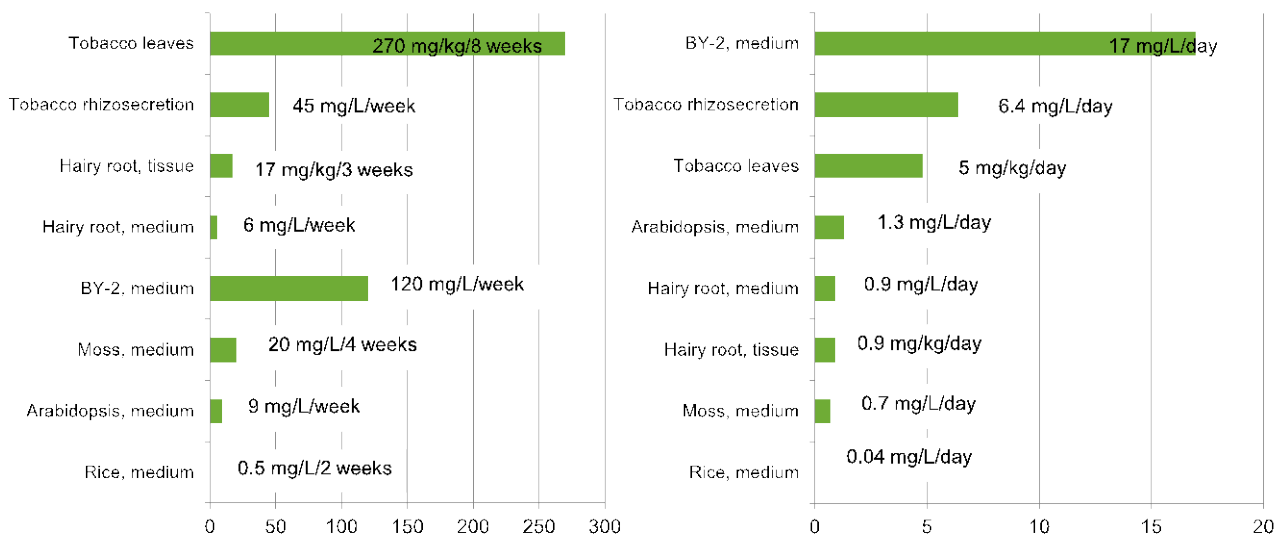
**Figure 2** Strategy to exploit existing (\* fast track) and newly-generated plant systems.

The aim of WP2 was to focus on the best-performing hydroponic, hairy root, moss and suspension cell culture bioreactor systems and to optimize extrinsic and intrinsic parameters, thus providing a consistent and optimal environment to maximize protein yields and ensure consistency. Existing (fast track) transgenic plants and cell lines were used to optimize the nutritional (task A) and physical (task B) parameters that affect the productivity and quality attributes of each system, and molecular factors (task C) within each system that could enhance yield and quality.

The aim of WP3 was to develop automated monitoring and maintenance systems for plant growth and plant cell/tissue cultivation, particularly non-invasive monitoring systems that can monitor plant health continuously, and either alert an operator if and when plants begin to show signs of poor health or disease, or automatically respond to signs of poor health by altering the physical and/or nutritional parameters of the contained system. Progress was made in the development of several of the planned automated technologies including systems based on chlorophyll and DsRed

fluorescence imaging for monitoring whole tobacco plants, hairy roots and suspension cells, a respiration activity monitoring system (RAMOS) for roots and suspension cells, and alternative systems based on the detection of scattered light as well as pH and O<sub>2</sub> fluorescence optodes. Work also began the use of the above systems to standardize growth parameters in each of the production platforms.

Work during the second 18 months of the project focused on completing the first three work packages and embarking on the remainder. The work in WP1 focused on the moss platform, which was the only incomplete aspect of the matrix of platform/product combinations, and the results from the remaining platforms were fine-tuned by testing product structure and activity. This allowed us to discontinue work on the species and platforms that performed less well and focus on those with the best productivity. The matrix of all CoMoFarm platforms was completed for the M12 antibody and this identified the most promising platforms considered in the project, as summarized in **Figure 3**.



**Figure 3** Comparison of all CoMoFarm production platforms in terms of productivity (left) and productivity over time (right) for the human monoclonal antibody M12.

The work in WP2 continued to optimize the cultivation parameters for the platforms not already tested exhaustively, including further testing of the nutritional parameters for the BY-2 cells, hydroponic tobacco plants, soil-grown tobacco plants and moss cultures, and the detailed characterization of physical parameters for BY-2 cells including the use of the RAMOS non-invasive monitoring device (WP3) to establish the health and performance of the cells during cultivation. We also completed the analysis of protease-knockdown BY-2 cell lines but found this strategy did not improve recombinant protein production. We also studied the effect of stacking optimized nutritional and physical parameters.

Rapid progress was made in WP3, including the further development of non-invasive monitoring devices for whole tobacco plants: a RAMOS device, an instrument allowing the real-time detection of DsRed fluorescence in tobacco leaves (which is particularly useful for the ranking of early seedlings to facilitate the selection of productive plants) and the GROWSCREEN-FLUORO system to determine the total projected leaf area and chlorophyll fluorescence. The RAMOS device was

also optimized for BY-2 cell suspensions and moss cultures, and integrated into prototype bioreactors by our industry partner Kuhner. These results allowed us to begin our main task of standardizing growth parameters, as required for the development of a GMP-compliant process.

The remaining three scientific work packages began in earnest, with initial work on protein recovery and purification from whole plants, cells and media (WP4) leading to the development of standardized downstream processing strategies that can be paired with the standardized operating procedures for protein production (WP6). This involved detailed product characterization, including tests for protein integrity, amino acid and N-glycan composition, as well as more in-depth analysis of protein functionality, all carried out as part of WP5. By the end of the third year we had made significant progress towards proof-of-concept for pilot-scale manufacturing using transgenic tobacco plants and the development of dedicated process equipment also suitable for the development of a cell-based production platform.

The planned duration of the CoMoFarm project was three years, but in order to finalize some delayed aspects of the project and investigate some additional opportunities, we requested and were granted a 7-month no-cost extension. The work undertaken during this time allowed us to complete the final experiments in WP1 (measuring the accumulation of M12 in rice suspension cells and T1 transgenic plants, and analyzing the functionality of the hemagglutinin produced in tobacco plants and hairy roots). With the matrix of platforms and products complete, five of the original seven platforms were taken to the next stage of the project, i.e. tobacco plants in soil, tobacco hydroponics (rhizosecretion), BY-2 suspension cultures, tobacco hairy roots and moss. A detailed comparison of all production platforms is provided in Table 1. In addition to these planned experiments, new experiments were carried out to determine the viability of hairy roots producing antibody M12.

In WP2, we completed the experiments to optimize the nutritional and physical parameters of the BY-2 and rhizosecretion platforms, by testing different cultivation temperatures, a 200-L orbital shaker and the impact of using BY-2 suspension cell lines with reduced protease activity. A novel method was established to produce monoclonal BY-2 cell suspension lines from a heterogeneous parental culture via the FACS-based selection of clones with high DsRed fluorescence. The performance of the best 'stacked' set of parameters for M12 antibody production in BY-2 cells was also tested, as well as the impact of high-osmolarity media on the rhizosecretion of antibody M12. We found that different formulations induced similar gains in yield, suggesting the improvement was caused by the higher osmolarity rather than the actual nutrients.

In WP3, the suitability of a non-invasive online oxygen monitoring system was tested when combined with an orbitally shaken 200-L disposable bioreactor, and in the hydroponic system we found that DsRed fluorescence does not correlate with the yields of secreted antibody and is therefore an unsuitable marker in this platform.

In WP4, the extraction and purification strategies were finalized for M12 produced in tobacco plants and BY-2 cells. A four-step process was developed for tobacco plants, comprising protein A affinity chromatography, CaptoAdhere chromatography, diafiltration and ultrafiltration. A three-step process was developed for tobacco BY-2 cells, comprising SP chromatography, protein A affinity chromatography and gel filtration.

In WP5, samples of antibody M12 from the different production platforms were tested for protein integrity and amino acid/glycan composition. As expected, the non-secreted M12 contained predominantly high-mannose glycans (the most common were Man7GlcNAc2 and Man8GlcNAc2) whereas the secreted version contained predominantly complex-type glycans (predominantly

Man3GlcNAc4Fuc1Xyl1). We also carried out a number of unplanned additional experiments on the bulk antibody product from the 200-kg scale process, not only confirming the integrity and purity of the antibody, and its functionality using vitronectin-binding studies, but also the signal peptide cleavage and the glycosylation profile of the final product and the amount of leached protein A and endotoxin contamination, both of which were found to be within tolerable levels according to regulatory guidelines. The sterility of the product was also tested by assaying for microbial growth on different nutrients media. The correct cleavage of the signal peptide from the M12 heavy chain was confirmed but the light chain produced two cleavage products (the correctly-processed product MASYVL was the most abundant).

In WP6, we tested the 200-L OrbShaker device for the large-scale cultivation of BY-2 cell suspension cultures (100-L volume). Cell growth and productivity in terms of antibody yield were comparable to the standard cultivation process with 150-mL BY-2 cultures in 500-mL shake flasks, thus achieving a 666-fold scale up without loss of productivity. The pilot-scale manufacture of M12 antibody using BY-2 cultures can therefore be achieved using the 200-L OrbShaker for upstream production in combination with the established M12 extraction and purification scheme for downstream processing.

**Table 1** Summary of progress at the end of the project – comparison of plant platforms for the production of antibody M12.

	Rhizosecretion	Tobacco plants	Hairy roots	BY-2 cells	Moss (H10 antibody)
Timeline for one production process (w/o purification)		8 weeks	3 weeks	2-4 weeks	4 weeks
Increased productivity through Medium optimization		comparison of different nutrient solutions and nutrient concentrations in hydroponics (FZJ: higher M12 concentration in non commercial nutrient solution)	30-fold increase in secretion with NAA and KNO <sub>3</sub>	12-fold (nitrogen-enriched medium)	6 fold (several components optimized)
Cell selection / screening		-	-	20% (flow cytometric sorting of monoclonal producer line)	50% (enlarged screen size plus coupled selectable marker)
Molecular approaches		-	-	overtransformation of BY-2 cells with protease knock-down did not result in the establishment of higher producing lines	-
Integrity after purification		confirmed	confirmed	confirmed	confirmed
SDS		confirmed (reducing and non-reducing Coomassie-stained SDS PAGE)	confirmed (reducing and non-reducing Coomassie-stained SDS PAGE)	confirmed (reducing and non-reducing Coomassie-stained SDS PAGE)	confirmed (reducing and non-reducing Coomassie-stained SDS PAGE)
Gel filtration		confirmed	not performed	confirmed	confirmed
N-terminal sequencing		performed	not performed	not performed	intended
Steps to get it ready for protein A		three-step filtration cascade	one-step filtration	cell removal by vacuum filtration, EBA	three-step filtration cascade, TFF-concentration
Functionality					
ELISA		confirmed	confirmed	confirmed	not intended
Purity		> 85%	-	> 98%	pending
Glycan pattern		high-mannose (ER-retarded) mainly complex glycosylation (apoplastic)	complex glycosylation: Three major plant glycan structures with no, one or two terminal GlcNAc sugar residues (in 1:1:1 and 1:2:6 rations in intracellular and secreted forms, respectively)	100% complex glycosylation	pending



	Rhizosecretion	Tobacco plants	Hairy roots	BY-2 cells	Moss (H10 antibody)
Costs per gram including protein A		~ €1,100,- (ER-retained M12 version at a productivity of ~400 µg/g FLW)	not performed due to low scalability (mg quantities can be achieved with moderate efforts and costs)	~ €13,500-7,200,- (secreted M12 at a productivity of 20 µg/ml)	calculation pending
Scalability		high	low	medium to High	medium to High
Evaluation of the production system		highly suitable for			
pros		cost efficiency, product quality	short production process	short production process, product homogeneity and quality	
cons		duration of one production process	low scalability	moderate productivities resulted in high production costs	

## **1.4 The potential impact and key dissemination/exploitation activities**

### *1.4.1 Impact*

By the end of the project we had evaluated four types of contained systems based on plants (whole plants in a hydroponic environment, hairy roots, moss bioreactors and plant suspension cell cultures) and submitted a full report on the yield and consistency of the same three target proteins achieved in each system, and their technological and economic constraints. We found that tobacco leaves and BY-2 cells were the most suitable platforms for the production of recombinant pharmaceutical proteins, based on the analysis of three candidates – influenzavirus HA (secreted and intracellular variants) and the monoclonal antibody M12.

With the best platforms identified, we analyzed the nutritional, physical and molecular parameters that affect protein yield, quality and consistency. Production is influenced by a wide range of variables and these were tested systematically to determine the best overall conditions for pharmaceutical protein production. For whole plant systems, we tested light intensity, photoperiod, temperature, hydroponic medium pH and composition, atmosphere, density of planting, and frequency of irrigation, with plants grown under conventional conditions (in soil) as controls. In the bioreactor systems, we tested various bioreactor designs and formats, fermentation strategies (batch, fed-batch or continuous), feeding rate, agitation, aeration, medium optimization and process optimization. We found that the most important conditions were the osmolality of the culture medium, the homogeneity cell and tissue cultures and the selection of elite producers during plant breeding, as well as the reproducible growth of plants, cells and tissues under controlled and optimized nutritional and physical parameters. The potential impact of these data will be twofold. First, they will provide useful parameters for the establishment of commercial plant-based contained production platforms, and second they will provide strategic guidance for the development and optimization of novel plant-based systems in the future.

Perhaps the greatest impact of the project is the development of automated systems for plant monitoring and maintenance. For large-scale production, it would be impossible to monitor plant growth manually and make appropriate adjustments to the environmental conditions analogous to the in-process monitoring routinely carried out with cultured cells. We therefore developed non-invasive monitoring devices based on DsRed fluorescence imaging and RAMOS that can be used to assess plant growth and health, and then if necessary make automatic adjustments to the environment of individual plants or the whole glasshouse. We have developed a working prototype which will have an immense impact on the industry because it will provide, for the first time, a rational approach for the standardization of conditions for the production of pharmaceuticals in plants. This will go hand in hand with the development of procedures for the optimized recovery and processing of each product, which is another critical aspect of pharmaceutical production. By considering the optimization and standardization of both upstream and downstream production we have facilitated the development of processes that are much more likely to comply with the regulatory framework governing pharmaceutical production. We also addressed the intrinsic and extrinsic aspects of product recovery simultaneously, by investigating strategies to improve protein secretion (to the apoplast in the case of hydroponic plants and to the culture medium in bioreactors), by studying different harvesting strategies (batch harvesting of leaves, milking and recovery from hydroponic medium, batch vs. continuous capture in the case of hairy roots and plant suspension cultures) and by developing downstream operations that are matched to these strategies. We also developed standardized assays to determine protein quality (integrity, functionality) which is also a necessary component of the regulatory dossier that must be presented to the competent authorities when developing a pharmaceutical production process.

#### *1.4.2 Key dissemination/exploitation activities*

The principal dissemination activities that have been carried out during the project include the publication of peer-reviewed original research papers and reviews (also see Section 2.1), the maintenance of a project website, the publication of popular articles and the presentation of data and meetings and congresses. Because most of the publishable data from the project has been collected in its final phase, the number of accepted publications is only moderate, but we have another eight manuscripts in preparation which will be submitted in due course.

The CoMoFarm project website was envisaged as a portal for the communication and dissemination of project information and scientific results both among the project beneficiaries and to the scientific community and public at large. The website has been regularly updated with important project information, including publications, standard operating procedures, information about our target proteins, presentations from project meetings and project reports (full reports available on the restricted-access part of the site, and publishable summaries on the home page).

The CoMoFarm project website also presents a summary slide deck outlining the project, and a dissemination article published by Research Media Ltd which can be accessed directly at the following URL: [http://www.comofarm.org/useruploads/files/p41-43\\_CoMoFarm.pdf](http://www.comofarm.org/useruploads/files/p41-43_CoMoFarm.pdf)

Data from the CoMoFarm project has also been disseminated by showing posters at conferences and by oral presentations at the following meetings and events:

- COST Action meetings in Gent 9-2011, Vienna 2-2012, Rostock 2-2013
- The Pharma-Planta Open Meeting and Final Consortium Meeting, Brussels, 25-10-2011
- GVC/DECHEMA 14–16-05-2012, Freiburg
- DAAD Chinese-German Workshop, Dresden, 21-9-2012
- PBVA, Verona 6-2013

## 1.5 Project website, principal contacts and list of partners

The URL of the project website is <http://www.comofarm.org/>

The principal contacts for the project are shown below:

Scientific coordinator	Project administration	Scientific officer
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A full list of the project partners and principal investigators is shown below:

1. Fraunhofer IME, Germany - Dr. Stefan Schillberg
2. Zürcher Hochschule, Switzerland - Prof. Dieter Eibl
3. Valtion Teknillinen Tutkimuskeskus (VTT), Finland - Dr. Anneli Ritala Nurmi
4. RWTH Aachen BioVT, Germany - Prof. Jochen Büchs
5. St George's Hospital Medical School, UK - Prof. Julian Ma
6. Plant Research International, Netherlands - Prof. Dirk Bosch
7. Forschungszentrum Jülich, Germany - Prof. Ulrich Schurr
8. greenovation, Germany - Dr. Andreas Schaaf
9. Dow AgroSciences, US/Germany - Prof. Wiltrud Treffenfeldt
10. Kühner AG, Switzerland - Dr. Tibor Anderlei