

Development of improved perennial non-food biomass and bioproduct crops for water-stressed environments

Deliverable 9.6

REVIEW OF PROJECT ACHIEVEMENTS FOR LONG-TERM IMPACT

Introduction

The overall goal of WATBIO is to improve the production of biomass under drought-stressed conditions, by providing important underpinning pre-breeding knowledge for both commercial and public plant breeding of three important second generation (non-food) biomass crops. This will enable biomass production on land that is not well suited to food production. WATBIO is committed to taking a strategic approach to supporting these impacts and is guided by an Impact Strategy developed in May 2013 and a long-term plan for prolonged impact from November 2015.

The purpose of this document is to review achievements in relation to these plans. This is based on statements of achievements provided by each Work Package Leader between October 2014 and December 2015. By 'achievement' we mean a substantial output or activity that contributes directly to the seven areas of impact identified in the aforementioned plans.

Major achievements in WATBIO to date that contribute to this outcome are:

1. Delivery of the project with no major deviations and with all deliverables and milestones intact, excluding minor variation.
2. Establishment of an extensive RNA-Seq and phenotyping dataset for highly contrasting wild material of *P. nigra* selected from across Europe.
3. Initiation on the largest poplar Genome Wide Association Study with Genotyping by Sequencing for 600 unique black poplar genotypes.
4. Delivery of a set of transgenic poplar, modified for candidate genes of interest for further analysis.
5. Establishment of over 12,000 poplar trees, at a commercial site, for GWAS and genetical genomics experimentation.
6. Development of the UK National Phenotyping Platform for assessment of a wide range of miscanthus genotypes for drought tolerance linked to RNA-Seq. transcriptomics in miscanthus.
7. Genetical genomics being undertaken in miscanthus F_2 population exposed to water stress.
8. Development of a unique mutagenesis screen in *Arundo donax* producing over 1,000 new genotypes of which 100 will be subjected to detailed analysis.

'Development of improved perennial non-food biomass and bioproduct crops for water stressed environments' is an international research project funded by the European Union's Seventh Programme for research, technological development and demonstration under the grant agreement FP7-311929



9. Development of new genomic resources at sequence level in arundo.
10. Identification of candidate genes for drought tolerance in miscanthus and testing using transgenic approaches in miscanthus.

In developing long-lasting impact from the project, several impact targets have been identified and these are:

1. Breeding of poplar. Impact Leader: Southampton University (Gail Taylor)
2. Breeding of miscanthus. Impact Leader: University of Aberystwyth (Iain Donnison)
3. Breeding of arundo. Impact Leader: Geneticlab (Daniele Trebbi)
4. Decision-making on crop development. Impact Leader (Bill Davies/Donal Murphy-Bokern)
5. Education and training. Impact Leader: University of Tuscia (Antoine Harfouche)
6. Scientific impact. University of Southampton, Impact Leader: (Gail Taylor)
7. Impact on the European Research Area. Impact Leader: Southampton (Gail Taylor)

The Impact Strategy was clear that much of the work to develop impact should be species-specific and this approach has been followed.

Delivering impact starts with embedding an awareness of the importance of wider impact in the research process. By complementing the supply of scientific ideas on the research side with demand signals from users of research, we aim to focus research on key technical outcomes. Therefore this review of achievement considers the research activities in that context.

The development of molecular tools and breeding techniques, drawing on basic research on model plant species, lies at the heart of the research in WATBIO. In particular, the timing of the project has meant that it has taken full benefit of the declining costs and long reads of DNA and RNA sequencing, afforded by Next Generation Sequencing. At the same time, outputs from WATBIO in the form of candidate target genes are available to take benefit from Genome Editing technologies already available in poplar. New germplasm better suited to droughted conditions will be identified in poplar, miscanthus and arundo. The research links novel molecular technologies focussed around next generation sequencing and emerging phenotyping platforms used for improving selection at an early stage in active breeding programmes. This biological research for enhanced breeding efficiency is complemented by environmental and economic assessments of the impact of the crops grown in field conditions across Europe.

Impact area 1: The genetic improvement of poplar

Figure 1. is a model of research and impact pathways in the research plan for the support of poplar breeding.

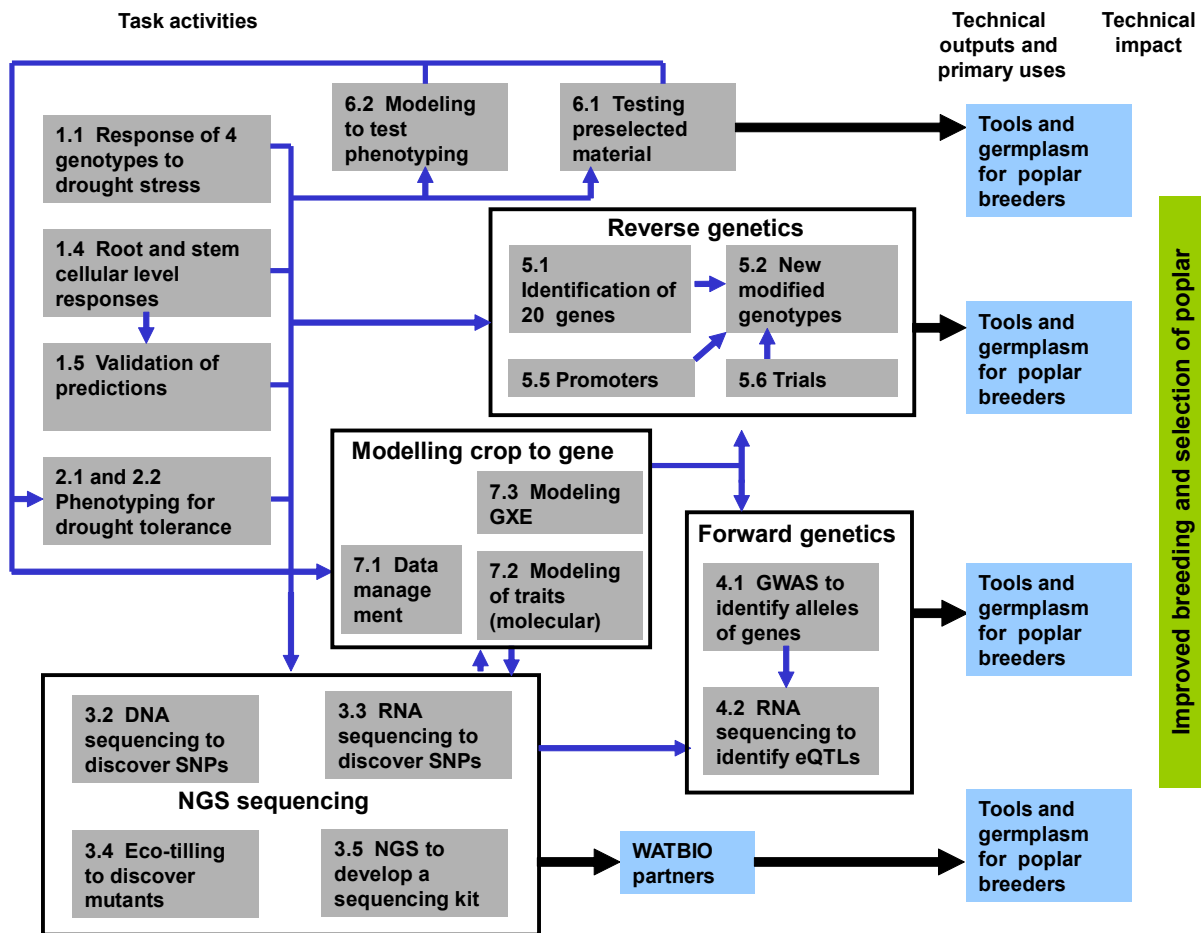


Figure 1. The relationship between research tasks and technical impact in the genetic improvement of poplar.

The primary impact of the breeding tools and materials is two-fold. Firstly, in our partner breeding organisation (Alasia) who can exploit the results in traditional breeding and selection programmes. Alasia has the largest commercial breeding programme of poplar in Europe and possibly, globally. Secondly, the tree biotechnology company SweTree Technologies is also world leading and offers an opportunity to commercialise outputs from reverse genetic approaches. Beyond these two WATBIO partners, the generic tools developed in WATBIO, such as transcriptomic data and functional and structural genomic resources will be made more widely available to other breeders and academic groups.

Phenotyping for a wide range of germplasm will provide new information on drought-stress tolerance available to breeders, while the selection tools developed will make the selection process more effective and more economical. Broadening the range of breeding material should result in greater durability of stress resistance and greater potential yield gains.

Precision phenotyping and genotyping will result in precision identification of genetic factors leading to selection tools for the breeders.

To support the breeding of poplar, the project has achieved the following:

Drought responses – root and shoot growth, gas exchange and the transcriptome

Leaf development

In Tasks 1.1, 1.4 and 1.5, three *Populus nigra* genotypes originating from habitats differing in water availability (“Spain”, “France”, “Italy”) were exposed to a well-watered control and to a moderate drought stress treatment for 5 weeks in greenhouse conditions. These ‘core’ genotypes were chosen to be representative of a wide-ranging set of more than 600 unique black poplar genotypes available to the project and obtained by partners in three other Framework Programme projects – POPYOMICS, ENERGYPOPLAR and EVOLTREE. We found constitutive differences in biomass productivity between genotypes, and a significant reduction in biomass accumulation under drought across all genotypes. Measurements of photosynthetic gas exchange revealed a genotype effect on transpiration rates per unit leaf area, net CO₂ assimilation rates, and stomatal conductance to water vapour. We observed differences in leaf and cell size, as well as stomatal index among genotypes, but limited G x E interactions were observed. Only the genotype from the arid region of Spain was able to maintain expansion of mature leaves under drought. However, growth of apical leaves under drought was maintained in the Spanish and French genotypes and stimulated under drought in the Italian genotype. These apical leaf expansion effects have been linked to altered cell production and expansion patterns. Bioinformatic analyses are underway to elucidate the molecular basis of this growth response. Multi-layered networks will allow the drought response to be examined in the course of leaf developmental stages.

Gas exchange and instantaneous water use efficiency

Drought increased water use efficiency, and some genotype effects have been explored in greater detail in a wider panel of germplasm during field imposed droughts at the commercial site in Italy. The genotypes differed constitutively in wood anatomical traits, and some of the analysed traits were also affected by drought. To link differences in phenotypic traits with changes in the underlying transcriptome, samples of developing xylem, fine roots, and leaves were collected for transcriptome profiling by RNA-Seq. Bioinformatic analyses revealed large differences in gene expression between tissues and genotypes. The extent of transcriptional remodelling under drought differed among tissues. By correlating the RNASeq data sets with phenotypic data, we were able to identify candidate genes related to drought-induced and genotypic variation in biomass and growth traits.

Moving beyond the three poplar clones studied above, new poplar genotypes were screened at a gravimetric facility at the University of Lancaster. The facility allows precise levels of stress to be applied while allowing access to plants for physiological studies. The studies revealed genotypic differences in the ability of poplar to maintain transpiration under drying soil conditions but also highlighted the experimental problems of establishing black poplar cuttings in such a facility and the impact that particular treatment regimes had on genotypic rankings.

The data from both core experiments of *Populus* have been used to develop a large RNAseq database alongside the development of more than 30 phenotypic measurements. The second phase of the project is to link these gene expression and phenotyping data to undertake network modelling analysis and to identify major gene hubs controlling drought response to contrasting genotypes of poplar selected from dry and wet sites and for which we have data for more than half a dozen tissue types.

Another important achievement is the generation of *Populus* × *canescens* ABA reporter lines, which can now be used to investigate ABA responses in planta. Additionally, *P.* × *canescens* cytokinin response reporter lines were exposed to drought stress, revealing interesting insights into cytokinin localisation patterns under drought.

Root growth

In Tasks 2.1 and 2.2, a method to analyse root growth kinetics of *Populus* was defined and successfully applied in an experiment where *Populus nigra* plants were exposed to a control treatment or osmotic stress. Growth rate response was monitored at whole root scale and at cell scale within the growth zone. Root apices were harvested from control plants, and after 0.5 h and 3 h of stress, and dissected to separate division zone and expansion zone. RNA was extracted and sequenced by IGATS (WP3). Root growth rate was significantly reduced 0.5 hours after the onset of osmotic stress, while the growth zone length was not affected. After three hours of stress, root growth was reduced to 30–40% of that of control plants. The growth zone length was also reduced but the division zone length was not. Transcriptome profiling by RNASeq revealed that, in both the division and the elongation zone, the gene expression response was strongly time-dependent, with double the number of regulated genes after three hours as compared to 0.5 hours of osmotic stress. Functional enrichment analyses revealed that among all differentially expressed genes, annotations related to hormone signalling and cell wall modifications were over-represented.

Genome Wide Association Studies (GWAS)

Following on from the development of a DNA genotyping chip in black poplar (Faivre-Rampant et al., 2016)¹, we are testing a new system to perform targeted genotyping-by-sequencing (GBS) in a cost-effective manner for poplar. This GBS resource for over 600 unique genotypes of poplar will be the largest of its kind for poplar in the world. The enrichment procedure enables targeting of specific regions or single SNP sites, enabling investigation of a pre-selected panel of *loci* with a genotyping-by-sequencing strategy. We developed the method starting with a new technology from NuGEN, a California-based biotech company. We use the same technology but with a different strategy: instead of mainly exploiting the “first-in-pair” read of each pair (as originally conceived in the NuGEN’s protocol) we fully lever the “second-in-pair” read, being juxtaposed to the probe oligonucleotide, specifically designed to be close enough to a known SNP. The strategy is aimed at maximizing the sequencing output to re-call selected SNP sites. This strategy will improve the overall power in the genome-wide association study enabling us to perform gene

¹ Faivre-Rampant, P., Zaina, G., Jorge, V., Giacomello, S., Segura, V., Scalabrin, S., Guérin, V., De Paoli, E., Aluome, C., Viger, M., Cattonaro, F., Payne, A., PaulStephenRaj, P., Le Paslier, M. C., Berard, A., Allwright, M. R., Villar, M., Taylor, G., Bastien, C. and Morgante, M. (2016), New resources for genetic studies in *Populus nigra*: genome-wide SNP discovery and development of a 12k Infinium array. *Molecular Ecology Resources*, 16: 1023–1036. doi: 10.1111/1755-0998.12513

space-centric genotyping with NGS technologies, where sequence information allows retrieval of extra information outside the target.

We have now sequenced approximately 600 samples by means of an enlarged set of more than 90,000 probes (*i.e.* 90k target SNPs). The sample pooling method has proved effective in providing uniform coverage across the samples. The bioinformatics pipeline implemented by IGATS retrieved genotype information for 68,287 of the chosen SNP sites along with extra genotyping of further 450,752 SNP sites.

The genotyping information provided by this method will provide unprecedented data to pursue whole-genome association studies in *Populus nigra*.

Field phenotyping – a major field phenotyping facility established

Alongside the genotyping efforts for GWAS, a major experiment, has been established with 6,000 trees in each of two experiments at the WATBIO field research site at Savigliano, Alasia (Italy) with a total of 12,000 poplar trees and several hundred arundo plants. This represents one of the project's most significant practical achievements. The site is equipped with extensive drip irrigation facilities, sensors and a weather station and all trees are grown to commercial standards. The poplar and arundo is maturing as the WATBIO research project progresses. This unique field research facility is now available to researchers outside the consortium. ALASIA has maintained these trees expertly and imposed irrigation treatments successfully. SOTON is engaging with other partners and collaborators (UIB, ULANC, CNR, ZIM, AU-IBERS and the University of Udine) to gain best use of this resource for the consortium, including for field testing of a wider range of genotypes that was possible in the 'core' experiment. The trees, now three years old, are the subject of an intensive programme of measurements and sampling under rain-fed and irrigated conditions. The second experiment led by UNITUS is also with poplar. A mapping F₂ population of 650 genotypes that are the siblings of a cross between two contrasting grandparents selected in northern and southern Italy is growing in eight replicates. The experiment provides an outstanding opportunity to assess NGS routes to genetical genomic approaches.

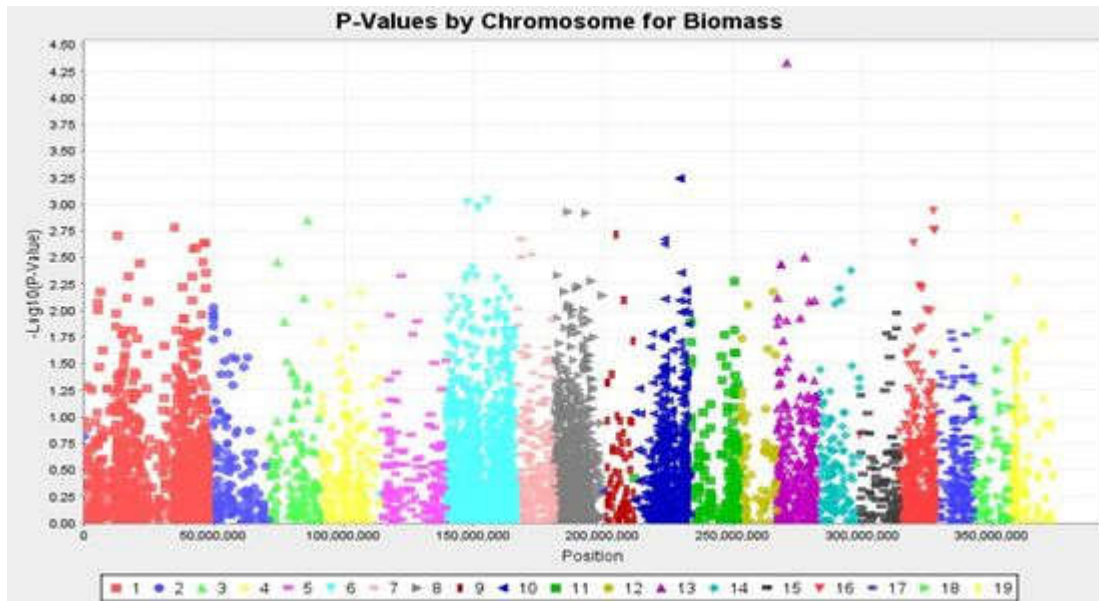


Figure 2. A manhattan plot showing the probability of individual SNPs being associated with biomass yield for the 2014 dataset under well-watered conditions. The SNP dataset is the 12K chip from energy poplar.

Alongside the measurements on the whole population for GWAS, we have conducted a detailed phenotyping effort (as a collaboration between SOTON, UIB, CNR, ZIM, ALASIA, ULANC and the University of Udine) on a subset of genotypes chosen to represent the full range of population variability. We found that this subset of genotypes exhibited distinct drought adaptation strategies as assessed by gas exchange and thermal imaging, pre-dawn and midday water potential, stomatal patterning, hormone signalling, carbon isotope discrimination and turgor loss point. This phenotyping effort which gives us a detailed picture of the physiological and biochemical basis for drought tolerance strategies.

Genetical genomics analysis of the genetic architecture of drought tolerance

UNITUS performed a time-course drought experiment in summer 2014 and 2015 on a full-sib F_2 population of over 500 *Populus nigra* genotypes (POP6) at the WATBIO field trial site in Savigliano. Parents and grandparents were also included in the experiment where trees were subjected to two irrigation regimes: well-watered and rain-fed (drought). At the onset of the experiments, morpho-physiological responses to drought treatment were measured. Two genotypes which were extreme with respect to their response to drought were used alongside the parents and grandparents for RNA-seq and subsequent eQTL analysis. Additionally, young leaves were collected from the extreme genotypes, parents and grandparents for DNA-Target-Seq that will provide high-quality SNP genotyping, a critical component of this genetical genomics analysis. The 2015 RNA and DNA extractions are underway and will be sent to IGATS for sequencing.

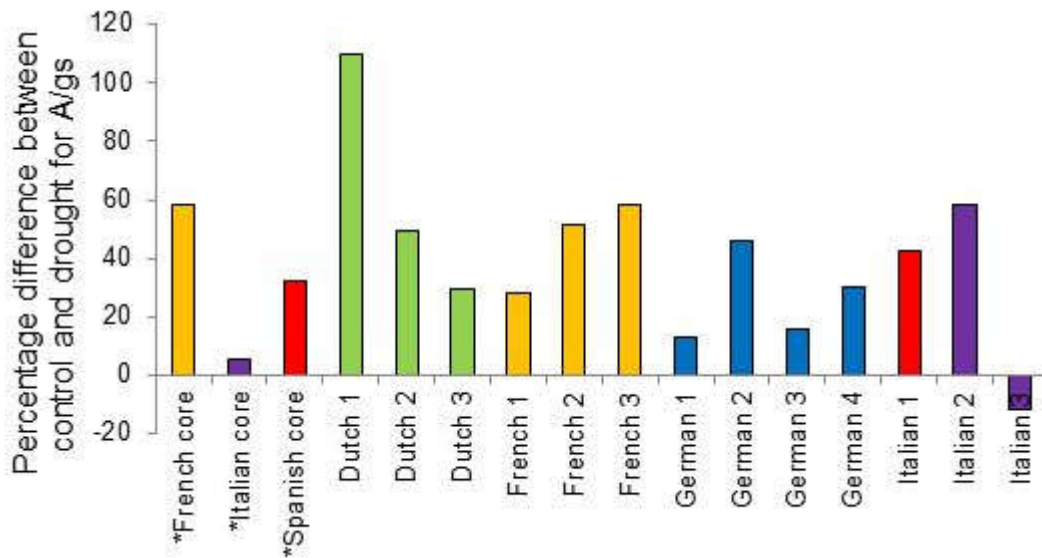


Figure 3. Variation in water use efficiency is evident in the diverse subset of 16 genotypes of *P. nigra* as measured by assimilation/stomatal conductance (A/g_s).

Reverse genetic approaches for improved poplar

The overall objective of WP5 is to identify and screen for potential candidate genes that can be used as tools to improve drought tolerance in poplar. This involves the selection of candidate genes using transcriptomic data set generated from core set of drought experiments. To date 10 genes have been identified and are being introduced into poplar. These genes are being expressed from constitutive and drought specific promoters. Moreover 3 promoters were tested for their ability to direct gene expression in response to drought stress in different tissues. More than 10 ABA signalling related genes were introduced into poplar and experiments to test the ability of transgenics to respond to drought stress is underway.

The RNAseq database has already been interrogated by STT, WU, SOTON and UGOE and a list of candidate genes supplied to STT for the development of RNAi and over expression (OE) lines in hybrid aspen, chosen because of its high efficiency and ease of transformation. 11 candidate genes and several constructs are being used to test these genes loci (WP5). The production of more than thirty original transgenic lines produced following a drought experiment and RNAseq analysis is highly novel and there is potential for the direct commercialisation of this material from WATBIO. Indeed, a two way agreement to protect one of these candidate genes is already being developed between SOTON and STT. Over the next phase of the project it will be timely to continue the research on these trees to cultivation out of culture, to pots and ultimately to the field. Already lines for ABA modification and signalling have been produced and these are being shared from SLU to UGOE and other interested partners. An open air semi-field experimental systems will be utilised for these studies (WP5).

Emerging commercially-relevant intellectual property

The poplar RNA-Seq data (WP1) and sequence data from a GWAS analysis in WP4 were provided to our commercial partner SweTree Technologies and other partners for the identification of candidate genes and the generation of transgenic plants with altered expression of these candidate genes. This is now underway. These results are relevant in terms of IP protection and exploitation. In addition, a more thorough re-analysis of the RNA-seq. data, alongside the phenotypic data from poplar is being shared with Wageningen University and is currently used for the identification of, for example, critical nodes in expression and phenotyping networks. These nodes represent influential genes of potential interest and will provide a further set of candidate genes to be developed beyond the life of the project, for exploitation in breeding.

Commercialisation of research on poplar

The Research Users Forum (RUF) is a starting point to ensure our commercial partners are fully engaged in the research and their specific needs are met. At the same time, we have ensured good two-way dialogue back from researchers on their achievements so that commercial partners are fully aware of the research activity. The WATBIO project has provided a supportive environment for translational research and genuine and long-lasting collaborative activities are now fully embedded in our activities. For example, the YARA ZIM probes have been tested in three separate experimental campaigns in droughted conditions. Valuable insight has been gained into the performance of these sensors on poplar from the project and adjustments to protocols made accordingly. For our breeding companies, Alasia, alongside transgenic approaches at STT, significant progress has also been made. For *Populus* a two way agreement is being prepared between STT and SOTON for IP protection of a gene loci underpinning yield with more likely to follow in the future.

Academic publications from WATBIO research on poplar

Allwright M.R. and Taylor G. (2016). Molecular breeding for second generation bioenergy crops. Trends in Plant Science 21: 43-54.

Faivre-Rampant, P., Zaina, G., Jorge, V., Giacomello, S., Segura, V., Scalabrin, S. Guérin, V. De Paoli, E. Aluome, C. Viger, M. Cattonaro, F. Payne, A., Le Paslier, M.C., Berard, A., Allwright, M.R., Villar, M., Taylor, G. Bastien, C. Morgante, M (2016). New resources for genetic studies in *Populus nigra*: genome-wide SNP discovery and development of a 12k Infinium array. **Molecular Ecology Resources – In Press**

Impact area 2: The genetic improvement of miscanthus

Figure 4 presents a model of impact pathways in the research supporting miscanthus breeding. The primary users are breeders.

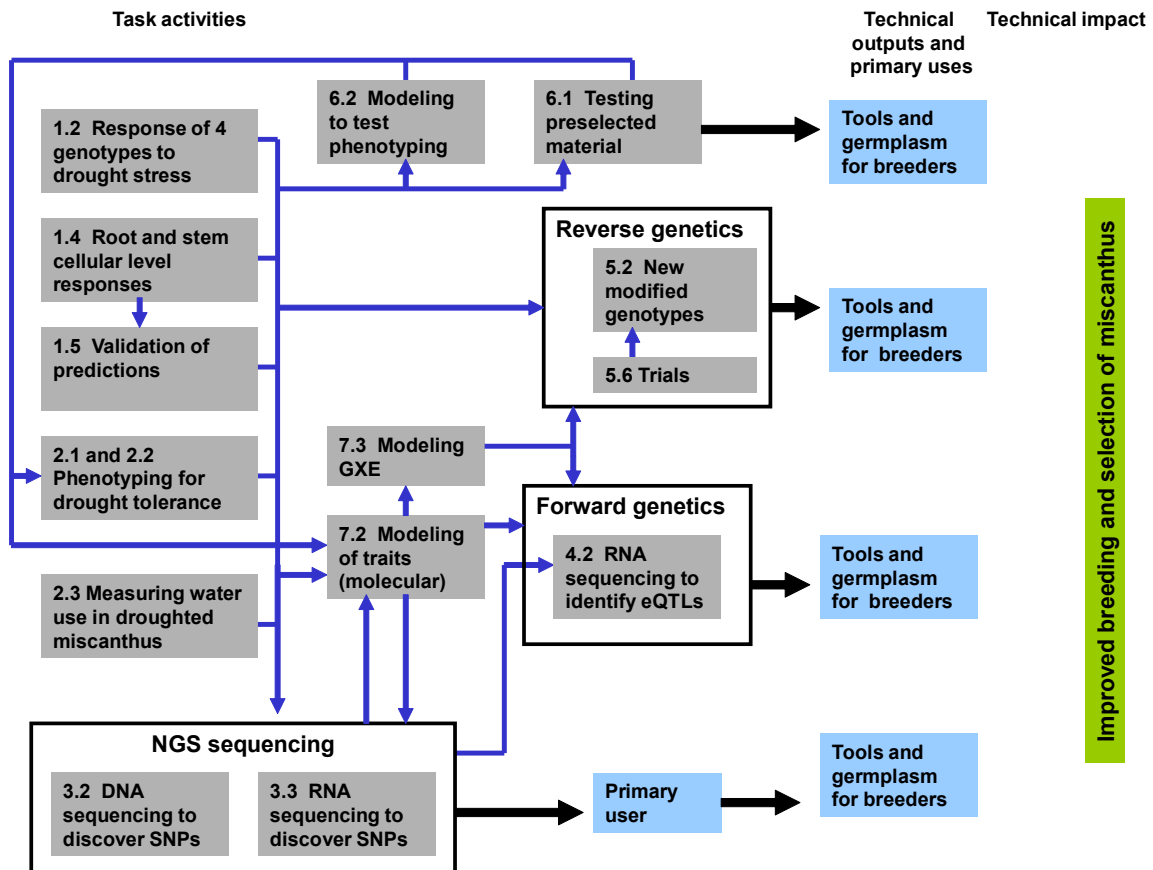


Figure 4. The relationship between research tasks and technical impact in the genetic improvement of miscanthus

The primary impact of the breeding tools and materials will be in the partner breeding organisation (IBERS) who will exploit the results in breeding programmes.

Germplasm identification will make new sources of drought tolerant material available to breeders while the selection tools developed will make the selection process more effective and more economical. Broadening the germplasm base through the use of novel materials identified should result in greater durability of stress tolerance and greater potential yield gains.

Improved phenotyping for drought tolerance

Precision phenotyping and genotyping will result in precision identification of genetic factors that are expected to give the desired phenotype, in turn leading to selection tools for the breeders.

The populations developed in WP1 are intended to enhance that precision through their size or genetic structure.

From Task 1.2, two core experiments with five miscanthus genotypes were successfully completed, one experiment where plants were exposed to drought and heat, and another where plants were only drought treated. The drought experiment was designed to be as close as possible to the *Populus* core experiments for cross-species comparisons. The plants were measured for growth and physiological responses. Elongation growth and aboveground biomass accumulation were reduced under drought, and the extent of this reduction differed between genotypes. The genotypes differed in biomass production per unit of water consumption under control conditions, and this ratio increased under drought. Samples for RNAseq and biochemical analyses were collected at two time points during the experiment. RNA has been isolated and sent to IGATS for sequencing, and data analyses will soon be completed.

Tasks 2.1 and 2.2 have screened around 100 novel miscanthus genotypes in the National Plant Phenotyping Centre at Aberystwyth. WATBIO has been fortunate to take advantage of this UK National Phenotyping Facility, based at IBERS UK. The genotypes are from the Aberystwyth collection. The plants were grown in a controlled glasshouse and treated with different degrees of drought stress while monitoring growth using an automated imaging system. The resulting growth curves allowed the measurement of two important traits: biomass accumulation and water use efficiency (WUE). The study revealed that these traits are generally negatively correlated in miscanthus; however, some genotypes were identified that maintained reasonable biomass accumulation and WUE under water stress.

Next generation sequencing

Sequencing of a number of RNA samples has been completed. Specifically, single reads, 50bp long, have been produced for the poplar and miscanthus core experiments with 7,491 and 5,261 million reads, respectively, summing up to 638 billion base pairs.

Genetical genomics analysis of the genetic architecture of drought tolerance

In Task 4.3, the miscanthus mapping population was removed from field sites and 870 plants were established in pots in order to determine the genetic architecture of drought tolerance in these genotypes.

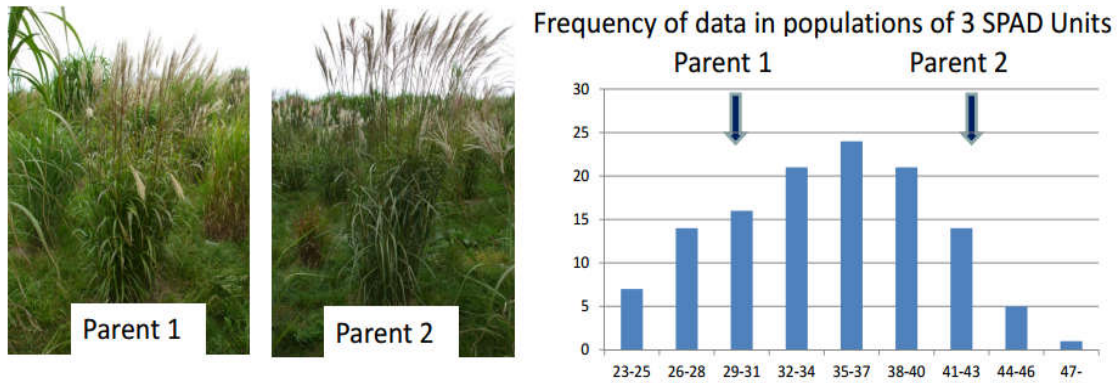


Figure 5. The parents of the *Miscanthus* mapping population (A). Chlorophyll content of the mapping population, with parent phenotypes indicated (B).

Various sampling strategies were discussed and we have focused on a random selection of genotypes from the population in excess of the number required and have completed a REML analysis of the elongation data as the most sensitive indicator of drought stress to allow extremes to be identified while accounting for experimental design. One hundred samples comprising 50 from across the range of responses from the drought treated plants and the equivalent 50 from control treatments will be ready for RNAseq analysis at IGATS in the next month. Due to the early flowering in the population the experiment could not be run in the first period of Task 4.3 therefore an extension (M50) was requested and approved.

Establishing the National Phenotyping Platform for miscanthus

Of particular international importance for the WATBIO project has been the establishment of the National Phenotyping Platform amongst the partnership (AU-IBERS) and where WATBIO has had priority access to this facility in which to conduct two drought experiment on several genotypes of miscanthus as part of the core experiments for this species.

Transforming miscanthus

Transgenic miscanthus is not widely available and limited success has been achieved on a transformation protocol with high enough efficiency of transformation to be useful. However in WATBIO, AU-IBERS are leading the way in this approach and now have a system running that they will test within the project using up to five constructs identified from the RNAseq data.

Commercialisation of miscanthus research

The exploitation of WATBIO research in the development of miscanthus is embedded in the miscanthus breeding and development programme at AU-IBERS. Given the role of AU-IBERS in

OPTIMA and OPTIMISC they are key partners to ensure the delivery and commercialization of miscanthus. Plant material has been bulked and supplied to the project and also used in the development of unique crosses and a QTL mapping population that is now being subjected to drought stress for the genetical genomic approach to elucidate candidate genes. A genotype has already been identified that produces more biomass under water-limited field conditions and this has now been established at two sites, in the UK and Germany for further study. This is complemented by a wider germplasm panel being assessed in the collaboration. The commercialization pipeline developed for miscanthus by AU-IBERS has been used as an exemplar for the commercialisation of arundo being planned by project partners and UNIBO and AU-IBERS will meet so that those at UNIBO can benefit from the experience with miscanthus in order to accelerate developments in arundo. This was made possible through the project WATBIO.



Figure 6. A field visit at the AU-IBERS Miscanthus Safari. A wide range section of the miscanthus development community attended the Miscanthus Safari at AU-IBERS in November 2014. This event was part of the wider effort on the commercial development of miscanthus.

Emerging IP for protection

The RNA-seq data from miscanthus and poplar is being shared with Wageningen University and is currently used for the identification of, for example, critical nodes in expression pathways. These nodes represent influential genes of potential interest in breeding programmes and have commercial potential.

Dissemination

Miscanthus research has been well represented at several international meetings, similar to poplar, with presentations at Plant and Animal Genome meeting, Dublin. In addition to a wide range of publications and presentations, the history of miscanthus development work, including the work in WATBIO, has been described in a recent paper now accepted in Global Change

Biology Bioenergy.² The work was also represented at the Biomass2015 meeting in Hohenheim in 2015 (www.biomass2015.eu).

Academic publications

Clifton-Brown, J. Donnison, I; Murphy-Bokern, D.; Robson, P; Pogrzeba, M.; Schwarz, K. (2016) Progress in upscaling miscanthus biomass production for the European bioeconomy with seed based hybrids. Global Change Biology Bioenergy (In Press).

² Clifton-Brown, J. Donnison, I; Murphy-Bokern, D.; Robson, P; Pogrzeba, M.; Schwarz, K. (2016) Progress in upscaling miscanthus biomass production for the European bioeconomy with seed based hybrids. Global Change Biology Bioenergy.

Impact area 3 – the genetic improvement of arundo

Figure 7 is a model of research and impact pathways supporting the arundo breeding. The primary users are breeders.

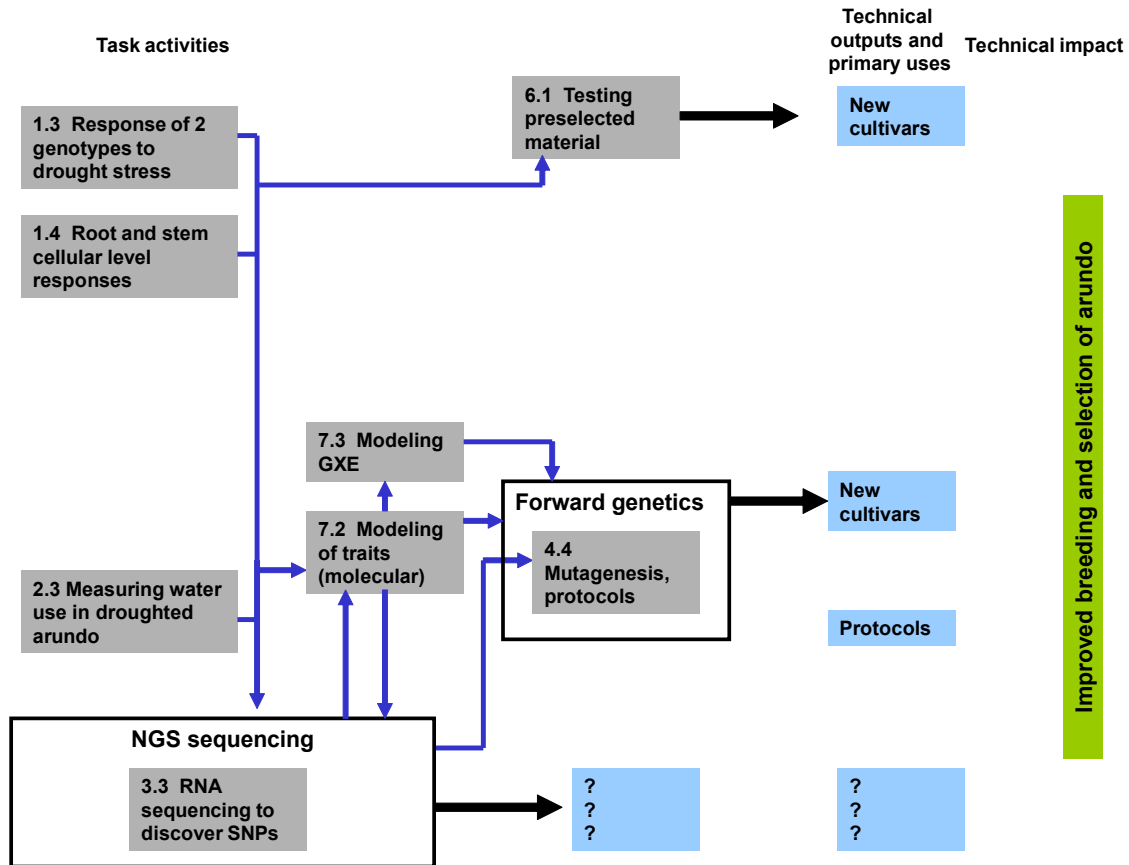


Figure 7. The relationship between research tasks and technical impact in the genetic improvement of arundo.

A pre-experiment using two arundo ecotypes originating from the contrasting environments of Italy and Morocco was completed successfully in September 2013. The two ecotypes showed very similar physiological responses to drought. Complementing this pre-experiment, a collection of 87 *Arundo donax* ecotypes was screened for growth and physiological traits. Based on these pre-experiments, one eco-type from CNR and three Alasia Vivai ecotypes were selected for the first arundo core experiment, which was conducted under field conditions in Northern Italy (at Alasia Vivai) in summer 2014. Due to high precipitation, the analysis of the plants’ responses to drought is limited and as a result this experiment was abandoned. As an alternative, and following consideration of potential for synergy between EU projects, an analysis of 40 genotypes under rain-fed and irrigated conditions was performed in late summer 2014 from the Optima (EU project ‘Optima’) collection of genotypes held by the University of Catania, Sicily. The most productive genotype from the 2014 field trial in Catania (genotype 6) was chosen for a new field

trial at the University of Catania where the OPTIMA genotype was grown alongside the WATBIO core genotypes from Morocco and Italy. The same three genotypes will be used in a pot based study conducted simultaneously in Firenze and Palma by CNR and UIB.

In preparation for the 2015 WATBIO arundo core experiment, CNR staff have visited Marrakesh, Morocco, and Etna, Sicily, to collect the *Arundo* ecotypes required for the study. This material has been distributed amongst WATBIO partners. The experiment will begin in June 2015 and we expect to present the first results at the conference Perennial Biomass crops for a Resource Constrained World (Hohenheim, Germany, September 2015), and the next WATBIO general meeting.

A protocol for the application of a split-root system for arundo was established. It was used to analyse physiological responses young plants to a pronounced drought.

A first pilot experiment of genotyping-by-sequencing was carried on arundo on 17 different samples using 100bp paired reads totalling 95 million reads equivalent to 9.5Gb of RAD (restriction-associated DNA) tags. The method showed to be informative in collecting polymorphic SNP sites across a panel of *Arundo donax* and *Arundo plinii* (a smaller-genome phylogenetically species related to *A. donax*) genotypes. Genotyping-By-Sequencing in arundo can also provide a lot of information on an unknown genus as arundo that might result very important in biomass production and highly drought resistant. Moreover, we are testing our GBS protocol to retrieve information on highly mutagenized arundo plants.

Physical mutagenesis of arundo

UNIBO and Geneticlab have successfully tested source tissue, radiation dosage and regeneration efficiency in arundo to establish a protocol for efficient physical mutagenesis based on both gamma-ray and Fast-neutron. This protocol has been used for large scale mutagenesis of *A. donax*. 1,014 *A. donax* clones have been regenerated from in-vitro cultures after treatment with gamma-ray at various dosages, while 100 *A. donax* clones were regenerated from in-vitro cultures after treatment with Fast Neutron at various dosages.

Trials of mutagenised plants have been established for phenotypic evaluation in the field and glasshouse. 1,000 *A. donax* clones from gamma-irradiation treatments were transplanted to the field on April 9–10 2015 (Figure 10) and 100 *A. donax* clones from the Fast Neutron treatments have been moved to large pots in a glasshouse for acclimation before establishment in the field in mid-September 2015 (MS 44 delivered, D4.4 in progress).



Figure 7. Transplanting c. 1,000 mutagenized *A. donax* clones on April 9, 2015 by GeneticLab and UNIBO.



Figure 8. Field observation of c. 1,000 mutagenized *A. donax* clones on Sept. 30, 2016.

Preliminary phenotyping screening of Arundo mutagenized clones.

The >1000 arundo clone field collection was phenotyped during 2015 and 2016 for major plant architectural traits affecting biomass production, namely maximum plant height, number of shoots, stem diameters. Additionally, observations about categorical (qualitative) traits such as leaf variegation and shoot architecture were collected. This investigation enabled to identify 93 putative mutants strongly affected for shoot vegetative traits. Additionally, several clones showing positive quantitative alterations in agronomically important traits (early vigor, erect habitus, high tillering) were observed. These clones will undergo further validation toward possible commercial exploitation.

Commercialisation

The possibility to patent the *A. donax* mutagenized clone collection is currently being jointly considered by UNIBO and GeneticLab. For *Arundo*, a two way agreement between Geneticlab and UNIBO has been signed to jointly own 1000 unique genotypes generated by the WATBIO project, as they become tested and commercialised as appropriate.

Academic publications

Physiological responses of *Arundo donax* ecotypes to drought: a common garden study.
 Haworth, M., Cosentino, S. L., Marino, G., Brunetti, C., Scordia, D., Testa G., Riggi, E., Avola, G., Loreto, F., Centritto, M. *Global Change Biology Bioenergy* (In Press).

Impact Area 4: Supporting commercial and policy decision-making

The project includes the environmental assessment of the cultivation of the improved crops and aims to contribute to better exploitation and socio-economic development of marginal lands and regions in Europe, particularly those already affected by adverse water stress conditions. This requires:

- a better understanding in farming and agri-business of the effects of perennial biomass crops adapted to drought-stressed conditions;
- greater appreciation in the policy communities of the impacts of increased production of perennial biomass crops in marginal areas; and
- increased understanding in wider civil society of the effects of perennial crops in these environments.

The users of this research are crop developers, planners and the policy community.

Assessment of the impact of drought tolerance

Field trials looking at the implications of drought tolerance for crop productivity have been established for the three species. An extensive laboratory and field analysis was undertaken on a wide range of poplar material that totals over 13,000 trees studied across a wide range of environments but all where drought tolerance for a set of agreed traits was determined. This provides a dataset of unprecedented significance for bioenergy crop breeding. A test of five miscanthus genotypes has established differences between genotypes in the majority of the parameters measured.

Experimental fields with 5 genotypes of miscanthus were tested in 3 different locations (2 in the UK and 1 in Balearic Island). The data gathered so far didn't allow the clear definition of the effect of drought on the divergent genotypes in different locations. It appears that the environmental effect is predominant on the genotype in determining the biomass production.

Efficient modelling of crop growth

A field trial within WP6 at AU-IBERS with 5 miscanthus genotypes maintained under two irrigation regimes with ten replicates. Preliminary measurements of soil VWC, EC, chlorophyll fluorescence and gas exchange have been conducted. Harvest of above-ground biomass completed in spring 2015. Trial continues to be monitored to compare this trial with its reciprocals in ULANC and UIB and the larger field scale trial at Braunschweig Germany.

Hydrological impacts

The biweekly monitoring of the water table changes under the root zone of *A. donax* and maize in a reclaimed field continued during 2014. Due to the wet and rainy season no contrasting changes were observed in either crop along the growing season. The average season water table depth under both crops was 1.9 m. This ongoing activity will continue during the next growing season.

Sensor technology

At the onset of the experiment and at three following data points, growth parameters (stem height and diameter, stem density), physiological traits (some parameters in collaboration with YARA

ZIM Plant Technology) and leaf biometry were measured, together with the cumulated biomass production at the end of the growing season.

The important issue for the project is whether there is a substantial genetics x environment x management (G x E x M) across our experimental sites. This would be a problematic but not unexpected outcome for us. In the last months we have developed a tool to assess the magnitude of this interaction. We have termed this tool 'a drought stress index'. This index allows interrogation of production data and calculation from these data of genotype production capacity and stress resilience as well as the impact of these variations on dry matter production on different sites.

Preliminary analysis suggests that genotype dominates over environment across different sites, i.e. the rankings for genotypes differing in production capacity and resilience are largely conserved across sites even though dry matter production varies with environment. **If this result is confirmed, this would have profound impacts on phenotyping practice and our capacity to speed up selection of genotypes for improved crop performance in dryland environments (a significant impact in the broadest sense of the word).**

Impact pathway 5: Education and skills

Work package 8 is focused on supporting long-term commitment of skilled scientists and technologists to the knowledge-based genetic improvement of these crop species. This has multiple effects that relate to all other impacts in the longer term.

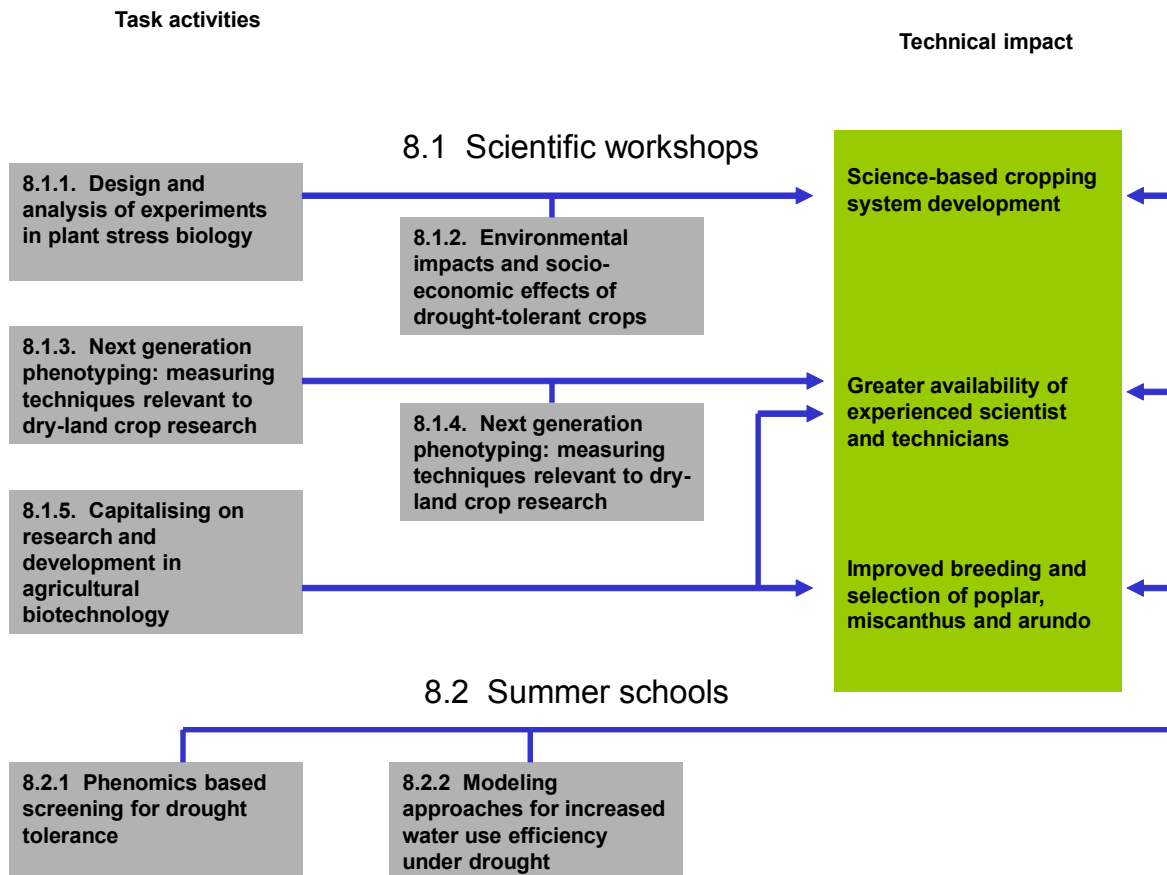


Figure 8. Impact pathways for workshops and summer schools

We strongly believe that our multidisciplinary approach that captures the whole value chain, from experimental design and data analysis, to environmental field physiology, to next generation genomics and phenomics, to systems biology and to commercial deployment of newly developed germplasm, can act as an exemplar for developing accelerated plant improvement approaches in future environments.

In addition to education and training, WATBIO Skills Workshops (four now delivered and a fifth in planning) provide an excellent way to ensure exchange of information and technical knowledge between university and industry experts with a very wide range of skills – for example, crop and tree breeder learning about molecular biology and physiology so as to establish a common understanding across the consortium of the range of research activities. This will ultimately help

industry partners to capitalize on WATBIO results and integrate science-based approaches into their breeding programs.

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Skills Workshops have attracted experienced participants who have provided their insights freely in the workshop setting. Workshop with industry/academic participation include the workshop on phenomics and high-throughput phenotyping, advances in imaging technology, standardisation of experimental protocols, environmental sensing and control, and data analysis. Such activities have led to increased cohesiveness and deeper understanding of phenotyping and genotyping drought stress in plants, next generation phenomics and high-throughput phenotyping, as well as phenomics data analysis. Dr. John Doonan, director of the UK Plant National Phenomics Center, Dr. Roland Pieruschka, Coordinator of the European Plant Phenomics Network, and Prof. Steve Hunt, CEO of Qubit Systems, Queen's University also attended the workshop. Dr. Hunt provided valuable input for the application of phenomics for the advancement of the project by explaining how to measure the onset and progression of stress.

Many of the impacts of education and training are intangible. The first WATBIO workshop was very relevant to the direction of the research project in addition to supporting education and training. It revealed opportunities to use WP8 as a strategic resource in the project underpinning the project's research planning and activities. Enabling early career researchers to participate in the project planning at this level also enhances the education benefits.

These Workshops have been particularly well attended by the 15 doctoral students who are working on the project.

Impact area 6: Scientific impact

WATBIO is a very significant investment in cutting-edge biological research. The systems approach ranges from molecular genetics, phenotyping, modelling (WP7) and environmental assessment provides a rich multi-disciplinary environment. This means the research has particularly high potential in terms of scientific impact and this impact potential must be exploited if the research is to fully deliver on requirements outside direct commercialisation by partners via the wider scientific and innovation community. Overall we plan to deliver over 100 peer-reviewed academic publications and 20 doctoral theses.

A significant number of outputs are either published or in preparation and add to the extensive series of presentations at meetings and other events in the form of posters and other dissemination activities. These are currently being put together into a database.

Impact area 7: Impact on the European Research Area

WATBIO brings together a wide diversity of skills not available in any single country and allows transfer of expertise from one crop to another. Many examples of this cross-fertilisation and European perspective exist across the project.

The development of arundo will benefit from the RNA-Seq and other approaches already being applied in poplar. The best systems for poplar transformation are in Sweden, whilst the world-class modelling platform for linking data is in The Netherlands. In addition, there are many shared resources in this project. WP1 relies on core experiments undertaken in a world-class facility in INRA France, but plant material will be sourced from UK, Italy and Germany.

Similarly in miscanthus, active breeding takes place in the UK, but requires bulking of material and yet the technologies developed from micro-propagation and bulking efficiently are in Germany. The field sites also range from south to north, providing valuable insight into G x E interactions. The project benefits from previous European-funded research that provides shared tools, expertise, shared learning and resources to be developed further in WATBIO including from EVOLTREE, POPYOMICS, ENERGYPOPULUS, DROPS, OPTIMISTIC, SWEETFUEL, GRASS MARGINS and OPTIMA.

Given the relevance of past and other on-going projects, the potential for synergy across projects was the subject of a special WATBIO internal report (D9.5). That revealed more opportunities than were anticipated and some very relevant strengths in other projects were identified in WATBIO. These include opportunities to use the synergies to support the generation of wider impact through breeding/crop development (WATBIO Impact Areas 1 – 3), policy support from economic and environmental assessments (Impact area 4) and education (Impact area 5), scientific publication (Impact Area 6), and the European Research Area (Impact area 7).

This work on synergy led directly to close interaction between the main relevant and (then) ongoing FP7 projects: Optima, Optimisc and GrassMargins. The four projects worked closely

together on the Bioenergy 2015 Conference held in September 2015.³ In addition to a Book of Abstract, a special issue of Global Change Biology Bioenergy and a Springer book of articles based on presentations, WATBIO worked with the other projects to produce an integrated summary of the state of the development of biomass crops.⁴

WATBIO's main field site is open to Europe's poplar and arundo researchers

The establishment of the WATBIO field research site at Savigliano (Italy) with a total of 12,000 poplar trees and several hundred arundo plants is one of the project's most significant practical achievements. The site is equipped with extensive drip irrigation facilities, sensors and a weather station. The poplar and arundo will mature as the WATBIO research project progresses. This unique field research facility is now available to researchers outside the consortium.

The site holds three field experiments. The first was designed by Dr Hazel Smith of the University of Southampton to examine approximately 600 contrasting poplar genotypes to support Genome Wide Association Mapping (see page 5). An eight block design is used and the trees, now 22 months old, are the subject of an intensive programme of measurements and sampling under rain-fed and irrigated conditions. The second experiment led by Professor Antoine Harfouche (UNITUS, Italy) is also with poplar. A mapping population of 650 genotypes that are the siblings of a cross between two contrasting grandparents selected in northern and southern Italy is growing in eight replicates.

Conclusion

The purpose of this report is to encourage the project partners to think about their work from the perspective of impact by identifying achievements that will drive impact. This is not as simple as it sounds in practice in a project largely focused on relatively basic biological research. This report supported an in-depth discussion about optimising impact as the project comes to a close.

The project is likely to generate very significant scientific impact. The conventional reporting of the research will generate most of the papers. The consortium has produced 34 peer-reviewed publications, 86 oral presentations and 30 posters. We can expect that conventional research reporting will double these. To complement these, it was concluded that the consortium should focus on highest impact synthesis papers. The consortium is in a position to make broader scientific impact in a number of areas, for example in developing the ideotype concept and the scientific basis for developing arundo, how trees might adapt to environmental change, drought adaptation in poplar, and the role of automated phenotyping in predicting field performance differences.

³ www.biomass2015.eu

⁴ Lewandowski, I., Clifton-Brown, J. and Murphy-Bokern, D. (2016). Developing perennial biomass crops for expanding European bio-based feedstock supply in a resource-constrained world. Summary of the Biomass 2015 Conference at the University of Hohenheim.

In developing impact through plant breeding, IBERS provides a model of how this type of innovation can be driven from a public research establishment.

For poplar, the research shows that transgenic lines generated in the project have effects on root growth, drought resilience etc. with the potential to find 1 or 2 trait gene combinations that can be protected (IP) and field trialled. These would be developed further including in other species using patenting for IP protection.

In the context of conventional poplar breeding, WATBIO has sequenced the genomes of 600 individuals, from which 40 strong candidate genes have been identified. We have the only poplar breeder in Europe (Alasia). WATBIO has the potential to move Alasia towards genomic breeding.

In arundo, mutagenesis has worked well, and the novel material has commercial potential. RNA seq. data are available to place a transcriptome in the public domain. As a crop, arundo is being domesticated and we now have pre-breeding information which will be useful in the future. Arundo as a crop has some commercial potential.

On education, WATBIO has provided an enriching environment for a large number of early-stage researchers and this is an important legacy. The lack of evidence of a strong GxE interaction for drought responses has long term implications for policy on developing marginal land. So far, no impacts on hydrology have been identified and therefore the impact as a source of biomass is dependent on the use of that biomass.