Boosting innovation in breeding for the next generation of legume crops for Europe

The plan for boosting the breeding of clover

Charlotte Jones, Germinal Horizon (GER), United Kingdom
Stig U. Andersen, Aarhus University (AU), Denmark
Jose de Vega, Earlham Institute (EI), United Kingdom
Andrew Griffiths, AgResearch (AGR), New Zealand
Stephan Hartmann, Bavarian State Research Center for Agriculture (LfL), Germany
Catherine Howarth, Aberystwyth University (ABER) United Kingdom
Anelia Iantcheva, AgroBioInstitute (ABI, Bulgaria)
David Lloyd, Germinal Horizon (GER), United Kingdom
Heathcliffe Riday, United States Department of Agriculture, (USDA) USA

Legume Generation Report 6

Legume Generation (Boosting innovation in breeding for the next generation of legume crops for Europe) has received funding from the European Union's Horizon Europe research and innovation programme under grant agreement No.101081329. It also receives support from the governments of the United Kingdom, Switzerland and New Zealand.

Legume Generation

Legume Generation (Boosting innovation in breeding for the next generation of legume crops for Europe) is an innovation action funded by the European Union through Horizon Europe under grant agreement 101081329. It also receives support from the governments of the United Kingdom, Switzerland and New Zealand. The Legume Generation consortium comprises 33 partners in 15 countries.

Disclaimer

Copyright

Citation

Please cite this report as follows:

Jones, C., Andersen, S. U., de Vega, J., Griffiths, A., Hartmann, S., Howarth, C., Iantcheva, A., Lloyd, D. and Riday, H., 2024. The plan for boosting the breeding of clovers. Legume Generation Report 6. Available from www.legumegeneration.eu and www.legumehub.eu

Contents

Background

A core principle of Legume Generation is that plant breeding is a species-specific entrepreneurial activity. Boosting breeding is therefore a species-specific effort led by innovators, i.e., those who take risks in innovation. To link the two worlds of public research and entrepreneurial breeding, six species-oriented breeder-led innovation communities (ICs) each link practical breeding with the supporting research-base in a transdisciplinary platform. This combines innovation and breeder focus on individual species/species groups in each IC with coherence and synergies in the provision of supporting science disciplines across the project. All six ICs innovate up to the point where newly-bred germplasm and tools are demonstrated on farm at technology readiness level 7. The support of the Clover Innovation Community is illustrated in Figure 1.

We will work with two species: red clover (*Trifolium pratense*) and white clover (*Trifolium repens*). The two species have different applications in farming, especially in sustainable grazing and mixed farming systems. Red clover is predominantly used as a forage crop (silage and hay), whilst white clover is used in mixed grazed swards. Both are important for nitrogen fixation in conventional systems and especially in organic systems. Despite this important role, genetic gains for key traits such as forage/seed yield and persistency have lagged behind that of the forage grasses with which they are grown. Both clover species are relatively undomesticated, and hence, there is scope to increase genetic gains relatively quickly. There is great diversity of agronomic relevance held in European clover germplasm collections.

The Clover Innovation Community

The Clover Innovation Community (IC) is built around four decades of partnership between Germinal Horizon (GER) and Aberystwyth University (ABER). Our aim is to improve the efficiency of clover breeding by developing pre-breeding material and methodological pipelines that can be used to produce elite commercial cultivars that overcome systemic production difficulties and that will benefit the sustainability of grassland agriculture.

This innovation community (Table 1) is led by Germinal Horizon (GER, UK), building on a long-standing collaboration with Aberystwyth University (ABER, UK). Phenotyping will be carried out by Germinal Horizon and Aberystwyth University, AgroBioInstitute (ABI, Bulgaria), The Bavarian State Research Center for Agriculture (LfL, Germany), and the United States Department of Agriculture (USDA). Genotyping will be carried out at the Earlham Institute (EI, UK), Aarhus University (AU, Denmark), and AgResearch (AGR, New Zealand). The Leibniz Institute of Plant Genetics and Crop Plant Research (IPK, Germany) and Radboud University (RU, Netherlands), respectively, will provide support on genomics and phenotyping.

The innovation community is supported by Donal Murphy-Bokern (DMB, Germany) as the science coordinator working with Amelie Detterbeck (Euroseeds, Germany) and Jasmin Karer (Donau Soja, Austria). They provide support on project integration, quality control, communications and business development.

As a group, the Clover IC has set achievable goals for work in both species, red and white clover, to be met within the lifespan of Legume Generation. These form a holistic approach to improvement in clover breeding. Work carried out in Legume Generation will inform germplasm selection for trait improvement and bring efficiencies to incorporating improved traits into commercial cultivars.

Table 1. Members of the Clover Innovation Community

First name	Second name	Role	Organisation
Stig U.	Andersen	Researcher	AU
Amelie	Detterbeck	Private seed sector association	EURS
Jose	De Vega	Researcher, data services	EI
Andrew	Griffiths	Researcher	AGR
Stephan	Hartmann	Researcher	LfL
Catherine	Howarth	Public-sector plant breeder	ABER
Anelia	Iantcheva	Researcher	ABI
Charlotte	Jones	Private-sector plant breeder	GER
Jasmin	Karer	Network	DS
David	Lloyd	Private-sector plant breeder	GER
Donal	Murphy-Bokern	Policy specialist; research director	DMB
Lars-Gernot	Otto	Researcher	IPK
Ivo	Rieu	Researcher	RU
Eric	Visser	Researcher	RU

Table 2. Number and description of pre-breeding and breeding programmes operated by Clover IC members

Partner	Pre-breeding	Breeding	Description
Germinal Horizon (GER)	5	5	Separate pre-breeding and breeding programmes for conventional small-medium leaf white clover, medium-large leaf white clover, hybrid white clover, conventional red clover and stoloniferous red clover
Aberystwyth University (ABER)	2		Pre-breeding programmes for white and red clover
Aarhus University (AU)	1	1	Pre-breeding and breeding programmes for red clover
AgResearch New Zealand (AGR)	1	1	Pre-breeding and breeding programmes for white clover
Bavarian State Research Center for Agriculture (LfL)	1	1	Pre-breeding and breeding programmes for organic red clover

Harnessing biology to boost breeding

The goal of the Clover Innovation Community is to take a scientific approach to direct funded research to improve the potential of new red and white clover cultivars. As with other agricultural crops, investment is key to successful breeding. Our aim is to improve the major agronomic traits in both species as well as providing novel sources of variation for abiotic and biotic stresses. Furthermore, knowledge attained within the project, particularly in the molecular area, will inform improvements in other forage legume species, e.g. lucerne/alfalfa (*Medicago sativa*).

We are using a diversity panel of wild and semi-wild accessions of white clover to identify germplasm that can thrive with reduced fertiliser application and perform well in diverse environments. In contrast to many arable species, clovers were domesticated recently (past few centuries) and cultivar development has relied heavily on ecotype selection. While this means that commercial cultivars are relatively unimproved, it also means that varieties are particularly amenable to improvement through hybridisation with landraces and wild populations. Modern genomic breeding strategies are highly underdeveloped. We aim to provide tools to substantially improve the efficiency of breeding. Quantitative trait loci (QTL) will be used to develop molecular markers for trait introgression into elite germplasm and models developed for predictive breeding to expedite cultivar development.

For both clover species, we are developing methodologies to harness heterosis. Red and white clovers are gametophytically self-incompatible species, meaning that plants with the same S-allele cannot fertilise one another. This mechanism restricts the number of compatible crosses within a population and diminishes genetic variability. A higher number of S-alleles in a population increases the likelihood of compatible pollen-pistil interactions, and ultimately the rate of successful pollination events, directly impacting seed set.

Consortium partners have previously found that specific combinations of S-alleles enhance hybridity and seed yield. It is hoped that this will provide a solution to reduced rates of seed production that have been observed particularly in red clover in recent decades, a factor that is seriously limiting the availability of improved varieties. We will also explore the possibility of using a semi-hybrid breeding methodology to facilitate the generation of F1 off-spring with these "enhancing" combinations of S alleles.

White clover (*Trifolium repens*)

Most white clover cultivars have a long commercial lifespan. Currently available commercial cultivars have been developed to thrive under historic farming practices. An example is the high inorganic nitrogen input typical of intensive dairy farming. Varieties were developed that could cope with high rates of fertiliser addition, which was to the detriment of clover's natural biological nitrogen fixation. Changing attitudes to fertiliser addition, led both by environmental concerns and rising costs, are having an impact on farming practice such as that the emphasis on nitrogen tolerance is shifting towards nitrogen fixation potential.

Moreover, commercial cultivars are frequently more susceptible to abiotic stress than wild germplasm due to their history of selection. In light of this context, we are adopting a pre-breeding strategy to identify white clover accessions that thrive with reduced fertiliser application and perform adequately in contrasting environments with different climatic variables. The focus is the improvement of our existing breeding programme at Germinal.

We will use multi-environment field experiments to characterise a panel of 200 white clover accessions. These 200 accessions of wild, semi-wild and commercial varieties of white clover are to be selected by the Clover IC based on their collection history to maximise panel diversity. Wild accessions are to be selected from as wide a range of European countries and environments as is possible, covering northern, central, southern, eastern and western regions. Commercial material will be selected from national and recommended variety lists and the European Common Catalogue, encompassing different leaf sizes and flowering times.

We will use whole genome sequencing technology to obtain population-based allele frequencies in the panel, and a genome-wide association analysis (GWAS) will be performed using the phenotypic data from the multi-environment field experiments. QTL will be used to develop molecular markers for the introgression of traits into elite germplasm. Furthermore, these results will be used to identify and select a panel of SNP markers that can be used in for the genomic-based prediction of breeding values, substantially expediting the selection pipeline.

Seeds from each line will be sent to Earlham Institute for germination and then DNA extraction for whole genome sequencing. The white clover diversity panel will be sequenced at the Earlham Institute using whole genome short read sequencing. A population-based approach previously used in the EU-Horizon project EUCLEG is to be followed, where 50 genotypes from each accession will be treated as one entry. This will provide information on the SNP fixated in an accession/population, but not on an individual genotyped basis. Elucidated SNP will be used in GWAS.

The white clover diversity panel will be sown in a randomised augmented plot design with controls for flowering-time and leaf-size in four contrasting environments. Two sites will be located in the UK: one under conventional farming practices; the other will be in a field with low phosphorus availability and will have no fertiliser input. Two sites will be established in Germany, both with standard fertiliser inputs: one in a dry area in Franconia and the other at higher elevation in the Alps.

Phenotyping will be carried out over three harvest years at each site, where assessments will include harvest yield, clover content, persistency both in yield plots and under hard defoliation (simulated grazing), leaf size, stolon density and number, crude protein and WSC . This will facilitate the selection of lines with greater persistence and tolerance to cold and drought conditions seen in parts of Europe, without impacting on yield and/or quality. Information on disease susceptibility will also be scored.

The Earlham Institute will provide the genotyping using a whole genome sequencing approach. They will run the bioinformatics to produce a SNP panel that will be used for GWAS at EI. GWAS, population structure and QTL and marker information will be passed to all partners of the clover IC. By understanding some of the molecular basis of important growth parameters, we will be able to integrate selected material more successfully into our breeding programmes.

We have standardised the traits to be measured on our white clover diversity panel. We have come to a reasonable compromise with essential characters to be measured, and a list of desired characters if we have time and manpower. Care was taken to ensure coherence in data so that the phenotyping supports GWAS.

Trial results and experimental designs will be deposited in the Grassroots web-based database maintained by EI. Similarly, marker panels and trait-association results will be stored in the marker web-based database developed by WP7. Both databases would be public later in the programme, and most information would be made public to other breeders.

The S-allele location and marker development in white clover is being carried out at AgResearch and Aarhus University. We are expecting that this will be finished before the end of the project, and so we will follow a similar process to that in the red clover, to use the process for improved hybrid seed production.

Red Clover (*Trifolium pratense*)

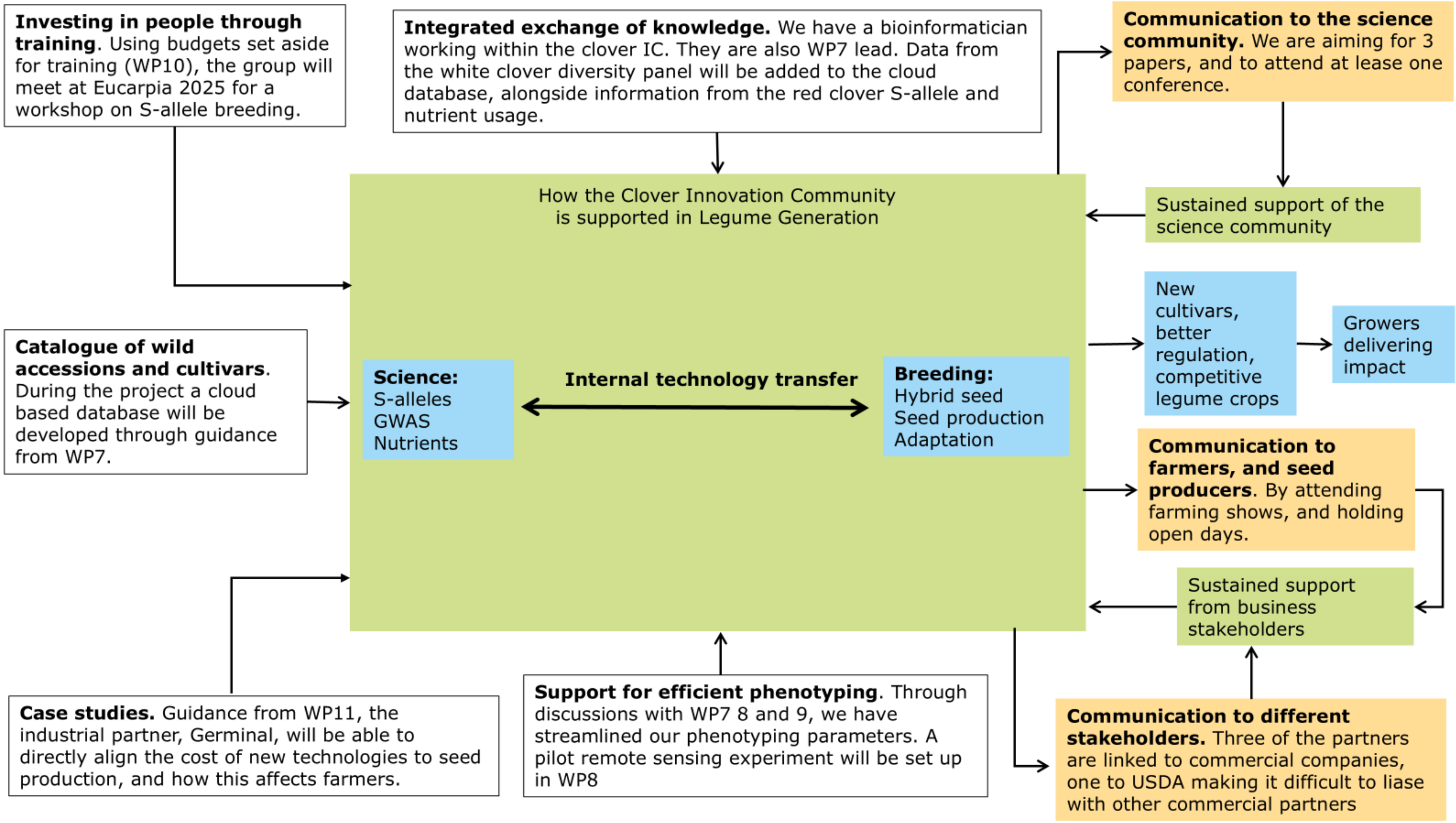
A significant and commercially limiting challenge for red clover seed merchants is unreliable seed production. Increasing commercial red clover seed production is a priority because it is a crucial link in the value chain of this forage crop. While elite varieties with enhanced characteristics are regularly being brought to the market, seed availability is limited and seed merchants have to substitute with older, outclassed cultivars, having a consequential effect on farm productivity and profitability.

Self-incompatibility, under the genetic control of the S-locus, limits the compatibility of pollen with a haplotype containing either of the two S alleles in the pistil genotype, preventing pollen tube development. Increasing the diversity of S-alleles in a population increases compatible pollen-pistil interactions, enhancing successful pollination and, thus, increasing seed yield. An existing microsatellite (SSR) marker system developed at USDA will be used to determine S-allele diversity within current commercial cultivars with disparate seed yield potential. Nine markers[1] will be run in a multiplex PCR reaction and sequencing performed on an ABI genetic analyser. This will enable us to assess the impact of S-allele diversity on seed yield potential. A panel of 100 red clover accessions will be assessed for S-allele diversity, followed by backcrossing of novel S-alleles into elite germplasm in both species, increasing S-allele diversity.

Further to this, consortium partners previously found that certain S-allele combinations boost hybridity, potentially increasing heterosis in the resulting F_1 progeny. A semi-hybrid breeding scheme will be developed within Legume Generation. Two parent F_0 populations, each limited to three S-alleles (distinct for each parent population) will be produced. When polycrossed, this theoretically produces an F_1 hybrid cultivar with 75% hybridity, increasing heterosis.

This semi-hybrid population will be used to examine whether this approach represents an improvement over standard synthetic population production, and whether sufficient uniformity is achieved to satisfy statutory mechanisms for the granting of Plant Breeders' Rights (PBR). Candidate material for producing the F_0 populations will be sourced from and grown at Germinal Horizon (Wales), Aberystwyth University (Wales), ABI (Bulgaria), LfL (Germany) and USDA-ARS (USA).

The seed yield issue will be further examined by testing the effect of boron application as a floral spray. Clovers are tolerant to low levels of boron in the soil; however, seed production is very sensitive to boron deficiency. There is also evidence that boron application can increase nectar secretion, which can increase pollinator visitation and improve seed set. This will be tested under glasshouse conditions through the use of clonally propagated genotypes applied with differing rates of boron to developing flowerheads. This will take place at ABI in Bulgaria and at Aberystwyth UK.



Investing in people through training. Using budgets set aside for training (WP10), the group will meet at Eucarpia 2025 for a workshop on S-allele breeding.

Integrated exchange of knowledge. We have a bioinformatician working within the clover IC. They are also WP7 lead. Data from the white clover diversity panel will be added to the cloud database, alongside information from the red clover S-allele and nutrient usage.

Communication to the science community. We are aiming for 3 papers, and to attend at lease one conference.

How the Clover Innovation Community is supported in Legume Generation

Sustained support of the science community

Catalogue of wild accessions and cultivars. During the project a cloud based database will be developed through guidance from WP7.

Science:
S-alleles
GWAS
Nutrients

Internal technology transfer

Breeding:
Hybrid seed
Seed production
Adaptation

New cultivars, better regulation, competitive legume crops

Growers delivering impact

Communication to farmers, and seed producers. By attending farming shows, and holding open days.

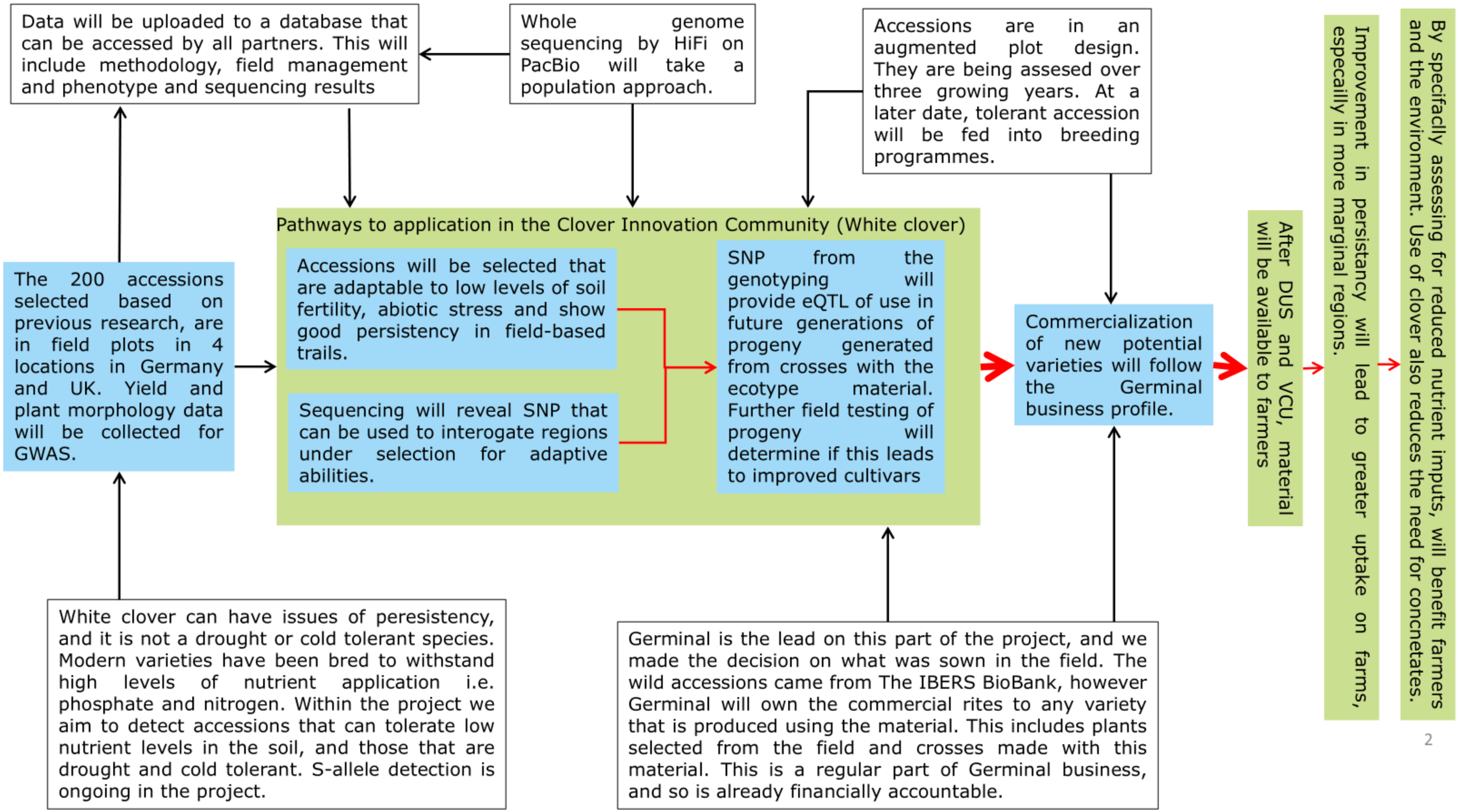
Sustained support from business stakeholders

Case studies. Guidance from WP11, the industrial partner, Germinal, will be able to directly align the cost of new technologies to seed production, and how this affects farmers.

Support for efficient phenotyping. Through discussions with WP7 8 and 9, we have streamlined our phenotyping parameters. A pilot remote sensing experiment will be set up in WP8

Communication to different stakeholders. Three of the partners are linked to commercial companies, one to USDA making it difficult to liase with other commercial partners

Figure 1. How the Clover Innovation Community is supported in Legume Generation



Data will be uploaded to a database that can be accessed by all partners. This will include methodology, field management and phenotype and sequencing results

Whole genome sequencing by HiFi on PacBio will take a population approach.

Accessions are in an augmented plot design. They are being assesed over three growing years. At a later date, tolerant accession will be fed into breeding programmes.

The 200 accessions selected based on previous research, are in field plots in 4 locations in Germany and UK. Yield and plant morphology data will be collected for GWAS.

Pathways to application in the Clover Innovation Community (White clover)

Accessions will be selected that are adaptable to low levels of soil fertility, abiotic stress and show good persistency in field-based trails.

Sequencing will reveal SNP that can be used to interogate regions under selection for adaptive abilities.

SNP from the genotyping will provide eQTL of use in future generations of progeny generated from crosses with the ecotype material. Further field testing of progeny will determine if this leads to improved cultivars

Commercialization of new potential varieties will follow the Germinal business profile.

After DUS and VCU, material will be available to farmers

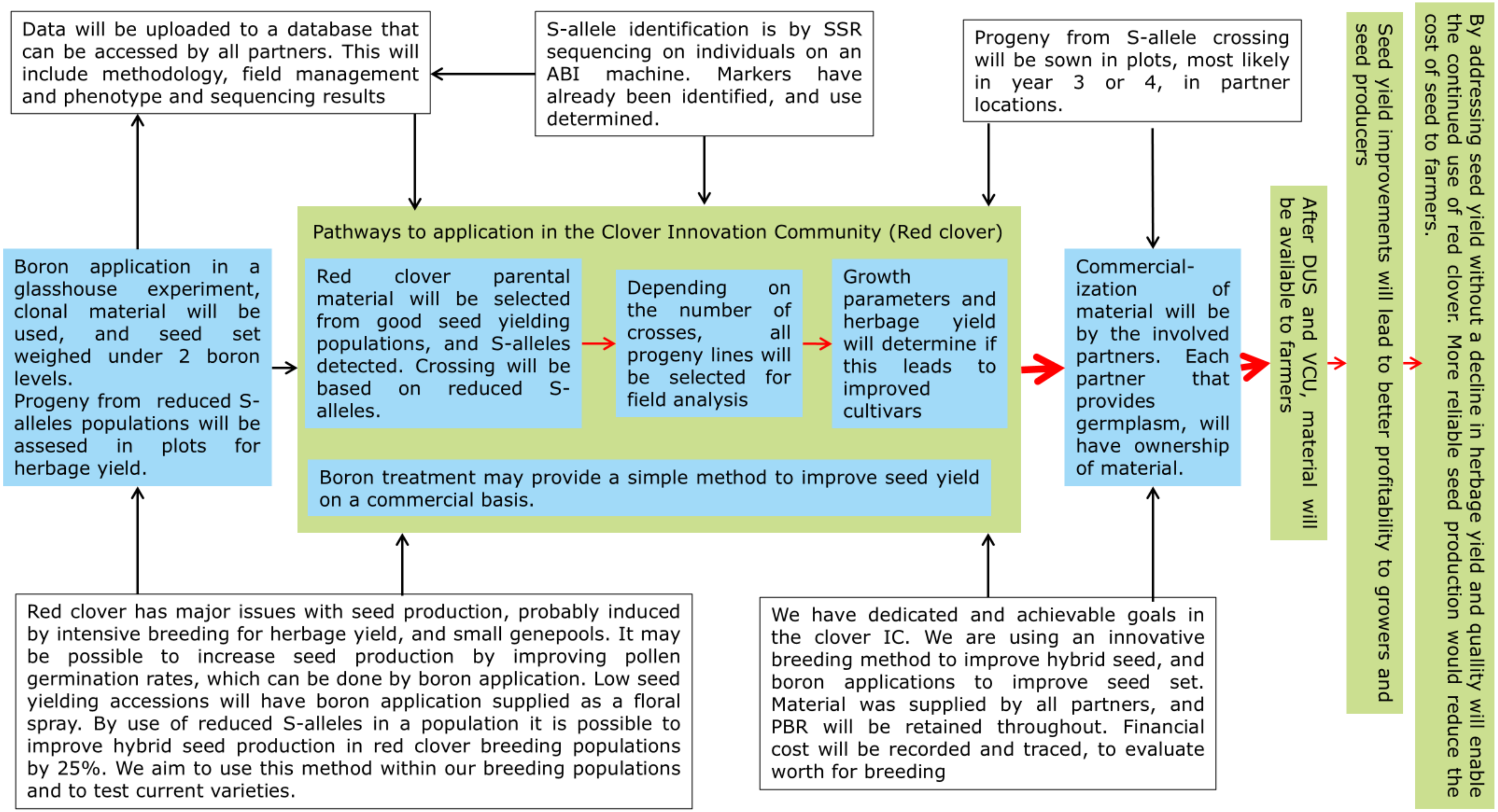
Improvement in persistancy will lead to greater uptake on farms, especailly in more marginal regions.

By specifaclly assessing for reduced nutrient imputs, will benefit farmers and the environment. Use of clover also reduces the need for concnetates.

White clover can have issues of peresistency, and it is not a drought or cold tolerant species. Modern varieties have been bred to withstand high levels of nutrient application i.e. phosphate and nitrogen. Within the project we aim to detect accessions that can tolerate low nutrient levels in the soil, and those that are drought and cold tolerant. S-allele detection is ongoing in the project.

Germinal is the lead on this part of the project, and we made the decision on what was sown in the field. The wild accessions came from The IBERS BioBank, however Germinal will own the commercial rites to any variety that is produced using the material. This includes plants selected from the field and crosses made with this material. This is a regular part of Germinal business, and so is already financially accountable.

Figure 2. Pathways to application in the Clover Innovation Community (White clover)



Data will be uploaded to a database that can be accessed by all partners. This will include methodology, field management and phenotype and sequencing results

S-allele identification is by SSR sequencing on individuals on an ABI machine. Markers have already been identified, and use determined.

Progeny from S-allele crossing will be sown in plots, most likely in year 3 or 4, in partner locations.

Boron application in a glasshouse experiment, clonal material will be used, and seed set weighed under 2 boron levels.
Progeny from reduced S-alleles populations will be assesed in plots for herbage yield.

Pathways to application in the Clover Innovation Community (Red clover)

Red clover parental material will be selected from good seed yielding populations, and S-alleles detected. Crossing will be based on reduced S-alleles.

Depending on the number of crosses, all progeny lines will be selected for field analysis

Growth parameters and herbage yield will determine if this leads to improved cultivars

Boron treatment may provide a simple method to improve seed yield on a commercial basis.

Commercialization of material will be by the involved partners. Each partner that provides germplasm, will have ownership of material.

After DUS and VCU, material will be available to farmers

Seed yield improvements will lead to better profitability to growers and seed producers

By addressing seed yield without a decline in herbage yield and quality will enable the continued use of red clover. More reliable seed production would reduce the cost of seed to farmers.

Red clover has major issues with seed production, probably induced by intensive breeding for herbage yield, and small genepools. It may be possible to increase seed production by improving pollen germination rates, which can be done by boron application. Low seed yielding accessions will have boron application supplied as a floral spray. By use of reduced S-alleles in a population it is possible to improve hybrid seed production in red clover breeding populations by 25%. We aim to use this method within our breeding populations and to test current varieties.

We have dedicated and achievable goals in the clover IC. We are using an innovative breeding method to improve hybrid seed, and boron applications to improve seed set. Material was supplied by all partners, and PBR will be retained throughout. Financial cost will be recorded and traced, to evaluate worth for breeding

Figure 3. Pathways to application in in the Clover Innovation Community (Red clover)

Legume Generation