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**Presenter Information**

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## Integration of traditional and biotech approaches to improve forage *Lotus* species for stressing environments in South America

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### Key points :

- 1 . Multidisciplinary approaches are required for understanding complex plant responses to the environment .
- 2 . Plant breeding can benefit from studies on plant physiology and metabolism and from high throughput methodologies like functional genetics and genomics .
- 3 . The concept of plant environment must consider not only edaphoclimatic conditions but also the biotic part of the environment , in particular plant-beneficial microbes .

**Key words :** Plant breeding , functional genomics , stress metabolism , beneficial microbes , multidisciplinary research

**Introduction** There is an increasing demand for high-quality animal-derived products worldwide . Livestock feeding rely mainly on grazing systems , but enhancing animal production is limited by the productivity , quality and extension of current natural pastures with grasses as predominant species . Pastures incorporating legumes have higher productivity and nutritional value for livestock production . In the Southern Cone of Latin America , over 45 million hectares of pastures could be improved using forage legumes . However , the alteration of fragile ecosystems determine that forage legumes are grown increasingly at the limit of their adaptation , in areas where the ability to survive periodic ( e . g . drought , flooding ) or continuous ( i . e . pH stress , salinity ) environmental stresses become an essential characteristic for success ( Graham and Vance , 2003 ) . In particular , the current pressure imposed by the expansion of intensive crop systems in South America has banished pastures areas to marginal , low fertility soils . Compared with cultivated *Trifolium* and *Medicago* species , several *Lotus* species have potentially higher adaptation to environmentally-constrained environments prevailing in the region .

Abiotic stress tolerance in crop and pasture plants is becoming increasingly important with global climate changes and other consequences of anthropogenic activities , such as the global increase in desertification , soil salinity , to name a few . One of the main challenges for breeders will be the production of superior germplasm that allows to maintain , if not enhance the current agricultural yields under increasingly adverse environmental conditions . Consequently , breeding programmes that aim to systematically improve general abiotic stress tolerance in plants are of prime importance for the sustainability of food production . Not only the increase in yield , but also in the stability of the production shall have a great effect on the productivity and the extent of current pasture areas needed to meet the current demands for more and better animal-derived foods .

The implementation of breeding programs , assisted by the latest knowledge and technologies , towards the development of improved cultivars adapted to environmental stresses , appears as a feasible strategy to increase the productivity of current grazing areas , essential for enhancing livestock production . Despite the well known adaptation of several *Lotus* species to marginal environments , private companies do not invest into breeding programs for these species , as these are costly projects targeted to ecologically marginal areas , with doubtful economic profitability in the short term . Therefore , improvement programs for *Lotus* pastures are usually isolated efforts implemented by public institutions with restrictive research resources . This paper provides an overview of the benefits of multidisciplinary research teams , the integration of molecular and metabolic techniques in plant breeding and the issues involved in the integration of beneficial microorganisms for restrictive environments .

**Genetic resources for integral research approaches** Genetic knowledge has speed up with the introduction of the concept of model plants . Among legumes two species , *Medicago truncatula* and *Lotus japonicus* have been chosen as plant models due to the close genetic relationship with agriculturally important legumes ( alfalfa , red clover , birdsfoot trefoil ; Fjellstrom *et al .* , 2003 ; Joulie *et al .* , 2003 ; Sato *et al .* , 2005 ; Young *et al .* , 2005 ) . Research with model legumes was initially centered on nitrogen fixation and symbiosis relationships , whereas model *Arabidopsis* approached plant important developmental processes . Furthermore , the research community has been involved in the development of germplasm stock centers for model and cultivated legumes , although the access to seed and information associated to the accessions is restrictively variable . In the last decade the international cooperation increased the genetic diversity of *L . japonicus* and *M . truncatula* with large collections of ecotypes , mutants , inter and intraspecific hybrids ( Niar *et al .* , 2006 ; Isobe and Akada , 2004 ) . Passport databases hold phenotyping information collected from individual and consortium research groups , rapidly increasing the potential value of the model germplasm and offering the opportunity to exploit the information for breeding of forage legumes .

Whereas research objectives in model species focus on genetic and metabolic issues , conventional breeding of cultivated species

has broad objectives associated to agronomic limitations . Forage breeding focus on specific traits associated with straightforward phenotyping such as disease and insect resistance , to extensive objectives related to universal restrictions such as persistency , forage productivity and quality , tolerance to stressful environments . Breeding for the latter aims has been restricted by the trait complexity . Additionally , the self-incompatibility reproduction mechanism and the natural tetraploid level of perennial legumes , together with the self-incompatibility reproduction mechanism ( e . g . *M . sativa* , *L . corniculatus* ) , results in populations composed of a heterogeneous mixture of highly heterozygous individuals , with high levels of genetic variation both within and between populations .

Breeding and selection for tolerance to abiotic stresses is restricted by the complexity of physiological and metabolic responses involved as well as the stress adaptation of the beneficial microorganisms . Although progress could be made in developing stress-tolerant cultivars of cultivated legume species using conventional breeding , the pace of this progress may be too slow to meet the growing needs of improving natural pastures , particularly when the task is developed by isolated institutions in developing countries . Progress in understanding physiological responses to stress , and further breeding advances leading to enhanced productivity and quality of crops and pastures , depend increasingly on multidisciplinary approaches ( Wuchty *et al .* , 2007 ) . Complex traits require the development of broad research partnerships and reinforce the challenge face by scientists involved in collaborative efforts based on multidisciplinary teams . LOTASSA , an interdisciplinary project financed by the European Commission , integrates 14 institutions and more than 100 scientists with the objective to raise levels of knowledge and to improve agricultural productivity for restrictive environments in the genus *Lotus* ( LOTASSA , 2005 ) . The project will take advantage of the close relatedness among forage *Lotus* species and the model legume *Lotus japonicus* to develop new and specific resources to assist and speed up breeding and selection of *Lotus* genotypes with improved adaptation to abiotic stresses ( water , salt and pH stress ) . LOTASSA will simultaneously provide inoculant rhizobial strains to guarantee optimal nitrogen fixation by *Lotus* pastures in constrained soils . One of LOTASSA ' s primary roles is to serve as a knowledge network sharing information based on standardized experimental protocols , as well as plant and rhizobia genetic resources generated by the Consortium . It uses the expertise of plant and bacteria geneticist , molecular biologist , biochemists , agronomists , and other professional specialists , to collect , analyze and disseminate data that aid breeding development . The information is made available using a number of different strategies , project meetings , publishing papers and reports , hosting electronic forum and providing *Lotus* germplasm and rhizobia databases on the LOTASSA web site . In this context , LOTASSA has also been playing an active part in disseminating information and promoting information exchange , establishing fora and specialized workshops .

The procedure to establish reference collections has been developed to a much more scale for model plants like *Arabidopsis* ( Meinke and Scholl , 2003 ) , where stock centers maintain and openly distribute experimental germplasm together with the genetic information . In a much smaller scale , LOTASSA will generate new characterized germplasm of cultivated and model *Lotus* species together with the characterization of symbiotic nitrogen fixing microbes under stress and corresponding databases for plant and microbes ( LOTASSA , 2005 ) . The purpose of these collections is to facilitate the exchange of plant and bacteria through a common material transfer agreement , maintaining and distributing the materials to ensure genetic uniformity in all partner laboratories . The application of biotechnology tools will yield benefits when they are inserted in a well structured breeding programme , which could make the best use through collaborative ventures with the formation of multidisciplinary teams within national/regional frameworks ( Robinson and Ruane , 2007 ) . Prerequisites for good genetic improvement are sound breeding strategy founded on traditional selection methodology and right breeding objectives . Thus , molecular and metabolic assist selection is viewed as a potential tool for plant improvement to be deployed alongside conventional phenotypic methods , a fusion that can be used to select the optimal variants in a population and one of the main issues established by the LOTASSA Consortium .

**The utilization of molecular markers in plant breeding programs for *Lotus* and other legumes** The genomic sequences of the model legumes *L . japonicus* and *M . truncatula* are currently determined at the Kazusa DNA Research Institute and the International Medicago Genome Annotation Group , respectively . At present a large part of the *L . japonicus* genome has been sequenced and 174 Mbp of BAC and TAC sequences were released into public databases . As part of this project 783 microsatellite markers were developed for mapping in a cross between the ecotypes Gifu ( that was used to develop most mutant populations ) and MG20 , which is the sequenced ecotype . This has led to the development of a genetic map ( Hayashi *et al .* , 2001 ) . Another genetic map was developed from a cross between *Lotus filicaulis* ( from Algeria ) and *L . japonicus* ( Sandal *et al .* , 2006 ) . With the use of the same microsatellite markers the two maps were found to be collinear . In order to cover the genetic diversity a third mapping population was developed from a cross between *L . japonicus* and *L . burttii* , which originates from Pakistan . Recombinant inbred lines ( F8 ) have been developed from these three populations and used for linkage analysis . These RILs are now also used for mapping of quantitative traits ( QTLs ) , which might be of agricultural interest ( Gondo *et al .* , 2007 ) .

*L . corniculatus* , *L . glaber* , *L . subbiflorus* and *L . uliginosus* are important agricultural species for the grasslands of South America . In order to transfer genome and genetic information from the *L . japonicus* model , microsatellite markers developed for *L . japonicus* , were tested in these cultivated species . We have shown that many of the primer sets will amplify a product in the agricultural *Lotus* species . The amplification products are often polymorphic between and within breeding lines and species . The number of successful markers transferred follows the expected evolutionary distance : *L . corniculatus* and *L . glaber* are

genetically closer to *L. japonicus* than *L. subbiflorus* and *L. uliginosus* and therefore more microsatellite markers can be transferred to the former species. Within the *Loteae* a very high degree of synteny (conservation of linkage) is expected and it is now possible to investigate this hypothesis using shared marker positions.

To further investigate the level of synteny within the whole legume family, the Stougaard's laboratory has developed a bioinformatics pipeline suggesting intron-spanning primers for general use in the legume family. These Leg marker primers are based on ESTs and genes from *Lotus*, *Medicago* and soybean and placed in conserved regions of single copy genes (Fredslund *et al.*, 2006a; Fredslund *et al.*, 2006b; Fredslund *et al.*, 2005). A number of primers were tested in bean and the distantly related legume genus *Arachis* and so far a total of 121 markers were developed for bean and 120 for *Arachis*. Apart from developing additional anchor markers in these species this also enabled us to look for synteny between *Lotus*, *Medicago*, bean and *Arachis*. For bean there is a very high degree of synteny to *Lotus* and *Medicago* (Hougaard *et al.*, submitted). For *Arachis* the level of synteny is as expected lower, but there are still several regions with synteny (Bertioli *et al.*, submitted). Because most crop legumes are closely related to the model legumes, and we have shown a high degree of synteny between them, it is very likely that knowledge obtained with the model legumes can be transferred to the crop legumes. One example of the use of synteny in legumes can be found in Hwang *et al.* (2006), where flanking markers considerably closer to a soybean resistance gene were found by thanks to synteny with *Lotus japonicus*. Given the close relationship between the model and the cultivated *Lotus* species the latter should be in prime positions to take advantage from combining the two approaches described here.

**Integration of metabolic studies in the LOTASSA project** Plant responses to environmental stresses involve a wide variety of metabolic pathways (Zhu, 2001). Salinity, drought and the combination of acidity plus aluminium are three important stresses that impose severe limitations for the expansion of agriculture to marginal soils in the South American cone. Understanding the metabolic responses to these stresses in model and cultivated *Lotus* species constitute is a major goal of LOTASSA.

Part of the responses of plants to salinity and drought consists on osmotic adjustment and sodium exclusion. Osmotic adjustment is achieved by the accumulation of compatible solutes capable of increasing cellular osmolarity, such as carbohydrates (trehalose, glycerol, mannitol), amino acids (proline, alanine), quaternary ammonium compounds (glycinebetaine, proline-betaine), etc. (Smirnoff, 1998). In addition, when available in the soil, potassium can be accumulated by plants leading to sodium exclusion. On the other hand, acid soils in tropical and subtropical regions frequently lead to toxicity of plants by a rapid mobilization and availability for plants of aluminium and other toxic metals. Salinity, drought and excess of metals lead to increase in the production of toxic free radicals and a decrease in antioxidant defences (Mitler, 2002).

Collaborative research incorporates evidence of partnerships for most research funding. LOTASSA project funded on this basis involves the authors and other 10 research institutions working with three different abiotic stresses to assist the adoption of more sustainable agricultural practices (LOTASSA, 2005). 7 different groups from 5 countries combine different expertises and facilities to address the metabolic responses of *Lotus* plants to different abiotic stresses within the LOTASSA project. The major metabolic processes under study include photosynthesis, photorespiration, water use efficiency, ion accumulation and transport, nitrogen metabolism (and in particular proline biosynthesis), polyamine metabolism and antioxidant defences. A combination of physiological, biochemical and molecular approaches is used, including enzyme measurements, metabolomics, transcriptomics, and the use of several mutant and transgenic plants as contrasting genotypes to determine the role of individual genes in relation to drought, salt and pH-Al stress. Functional genomic tools as TILLING (target-induced local lesions in genome), developed at the John Innes Institute (United Kingdom), are being used for this latter goal.

So far, more than 15 different physiological parameters, 40 enzymes and around 40 different ions or metabolites were individually analyzed in leaves and roots of several model *Lotus* species (*L. japonicus* MG-20 and Gifu ecotypes, *L. filicaulis*, *L. burttii*) and different cultivars of the cultivated *Lotus* species as well (*L. corniculatus*, *L. glaber*, *L. uliginosus*). In addition, a comprehensive list of hundreds of genes and metabolites highly responsive to stress were also found from transcriptomics and metabolomics respectively, and are now under study. As a result of these joint studies it is expected to obtain a clear picture of the main metabolic changes associated with different types of stress, which may assist for breeding programmes aimed to enhance salt, drought and pH-Al stress tolerance in *Lotus*.

**Integration of rhizobiology into plant breeding for constrains environments** It is quite common that plant genetic research or plant breeding be developed in connection to detrimental plant-interacting organisms (microbial pathogens, insects, etc.). In most these cases, existing or newly generated plant genotypes are selected for rather well defined characteristics: partial or total resistance to the invading organisms. But there are rare examples of integrating plant and microbial selection for maximizing beneficial plant-microbe interactions (BPMI). Beneficial microbes are those that help plant growth and development, either by facilitating the acquisition of essential nutrients, modifying hormonal balance or helping plants to fight against pathogenic organisms. Most beneficial microbes, such as symbiotic nitrogen fixers, mycorrhizal fungi and many other rhizospheric microorganisms interact with the plant root system although their effects are visible in all plant organs and may drastically change the plant physiology and even organ morphology. Besides, BPMI strongly rely on the environment and on both plant and microbial genetic diversity, as there is usually a certain degree of genotype-to-genotype specificity. Therefore, combining

plant selection with optimal microbial performance is a difficult task due to the complexity of the interactions involved . But it should be possible to integrate the development of novel , improved plant genotypes with the simultaneous selection of those beneficial microbes that optimally interact with the newly arising plant varieties . This should be feasible under either of these situations :

When breeding involves well defined plant agronomical traits , selection should be performed including the beneficial microbes in the plant growth environment . For instance , many experts agree that most terrestrial plants do not actually have roots but have mycorrhiza , and therefore experimental selection should always be done with mycorrhized plants .

When breeding for complex characteristics determined by multiple genes , such as tolerance to abiotic stresses , microbial selection for similar traits may be done in parallel to plant selection , with final rounds to establish the most efficient plant-microbe combinations .

When breeding for improved interactions with microbes , simultaneous selection of the most competent plant and microbial genotypes may be necessary . Even complex traits such as symbiotic nitrogen fixation can be successfully bred for ( Herridge and Rose , 2000 ; Graham *et al.* , 2004 ) . More importantly breeding without considering beneficial microorganisms may lead to progressive loss of the plant capacity to benefit from the microbes ( Kiers *et al.* , 2007 ) .

Within the project LOTASSA , researchers recognise the importance of symbiotic nitrogen fixation for the success of *Lotus* pastures in environmentally-constrained areas . Therefore , existing plant and rhizobial resources are screened in a way that the most reliable , the best performing plant-microbe combinations in stressed South American soils are selected .

**Conclusions** In collaborative research involving plant and microorganism genetic resources the aim should be to identify how best the partnerships might be facilitated to promote knowledge sharing . Many issues that emerge in international projects are common , including the need for mutual commitment between research groups and the incorporation of local knowledge of existing genetic resources . Participatory approaches are commonly interpreted as processes for farmers plant breeding . Yet , integrated projects show that participatory research approaches can increase adoption of molecular and metabolic knowledge into applied plant breeding , as well as improved plant and beneficial microorganisms interactions . This contribution of knowledge among specialized researchers , and the opportunity to reconcile their different interpretations of results , is what underpins the universal appeal of participatory research for people learning to implement change .

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