

## LUCERNE BREEDING IN EUROPE: RESULTS AND RESEARCH STRATEGIES FOR FUTURE DEVELOPMENTS

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### SUMMARY

Lucerne breeding efforts in Europe are analysed from 1995 to the present considering three main topics: (i) germplasm collection, conservation and evaluation; (ii) genetics, breeding and genetic transformation; (iii) forage quality. The importance of European germplasm resources has been clearly evidenced with special interest for material which could be useful to add new traits such as grazing tolerance and resistance to stresses to cultivated lucerne. The results of genetic and breeding activities are more relevant in the area of modern biotechnologies and basic research than in the area of variety development, the most recent varieties being characterized by a forage production which is on average only 5% higher than old varieties and adapted ecotypes. The reasons for this slow progress are discussed. Transgenic technology is giving interesting results but, at the moment, the negative attitude of European public opinion toward genetically modified plants and the possibility of gene flow from transgenic lucerne to cultivated or wild populations make it difficult to hypothesize the release of genetically modified lucerne in the near future. An interesting evolution of lucerne breeding has been evident in the last few years from the efforts aimed at creating more digestible varieties with high agronomic performances.

**Keywords:** Germplasm, genetics, breeding, transformation, selected traits.

### INTRODUCTION

Cultivated lucerne (*Medicago sativa* L. subsp. *sativa*,  $2n=4x=32$ ) is a species native to the Middle East and the most common forage species in the world. In a large part of Europe, it is the most important forage legume, and is grown for hay, dehydrated forage, pellets and silage. Lucerne is also especially important for restoring soil fertility: with no cost for nitrogen due to N-fixation by *Rhizobium*, the residues increase soil organic matter, the root system mobilizes nutrients deep within the soil profile and the soil

structure, permeability to water and water retention are improved. Furthermore, lucerne only requires low inputs of herbicides and pesticides and no N fertilisers. This agrees with EU policies and public concern about the environmental impact of agricultural activities. Lucerne hay fields were also shown to be highly favourable to biodiversity enrichment. Its high protein content meets the requirements of the feed market, particularly after the problems caused by the use of concentrates of animal origin, presently forbidden in the EU (Torricelli *et al.*, 2003).

In approaching the general theme of the review, papers published since 1995 have been divided into three main topics: A. germplasm collection, conservation and evaluation; B. genetics, breeding and genetic transformation; C. forage quality. Even if several papers could be included into more than one topic, we collected 26 papers in A, 119 in B and 23 in C. We subdivided the large B group into: B1. genetics and cytogenetics (36 papers), B2. breeding, variety development, identification and evaluation (65 papers) and B3. genetic transformation (18 papers). In the following pages, we summarize what was obtained.

## **A. GERMPLASM COLLECTION, CONSERVATION AND EVALUATION**

With regard to this topic the experiences of French and Spanish researchers working on natural Spanish populations of lucerne named Mielgas deserve particular interest. Stock rearing is a traditional component of Mediterranean agriculture systems and nowadays it also represents the best way to valorise population-drained marginal areas for agriculture, landscape maintenance and spare-time activities (Delgado Enguita, 1989; Ben Chaâbane, 1997; Prosperi *et al.*, 1999). As a consequence, plant breeding activities in these areas must be targeted to obtain lucerne varieties adapted to grazing and resistant to environmental constraints. A prerequisite to reach the target is the collection and evaluation of local germplasm. Since the second half of the eighties more than 100 natural populations of Mielgas have been collected from Spanish pastures and evaluated under both pasture and cutting managements. Most populations were characterized by a prostrate habit and a great ability of soil colonisation. They showed a wide variability for growth rhythm (for example, the first cut Dry Matter Yield, DMY, ranged from 39% to 75% of the total DMY depending on the accession) and, on the whole, better persistence with respect to the control varieties, fast spring regrowth and interesting seed yield.

The different behaviour of the Spanish natural populations with respect to the maintenance of their own distinctiveness when grown in close proximity to lucerne crops established with seed belonging to cultivated ecotypes or bred varieties makes the issue of gene flow from cultivated to wild plants of critical importance. The effects of gene flow have been studied with the use of quantitative traits, allozymes and molecular markers (Jenczewski *et al.*, 1999). Some Mielgas populations appeared to be intermediate between

wild populations and cultivated ecotypes; as a consequence, gene flow from cultivated to wild populations is clearly present, resulting in the formation of hybrid populations. Some other Mielgas populations seem to be able to maintain themselves completely separated from the neighbouring cultivated ecotypes as a probable consequence of time, spatial and reproductive isolation. Differences among natural and cultivated populations are more evident for agronomic traits than for neutral markers. This can be the consequence of a natural selection pressure that, at least in some locations, is acting against the gene flow which tends to transfer agronomic traits typical of cultivated material to natural populations. On the whole, Mielgas germplasm is very interesting for the presence of different positive traits such as prostrate growth habit, persistence, tolerance to water stress, a good level of tolerance to stem nematode and interesting seed production, but needs to be selected at least for total DMY and for vegetative growth in autumn. The work done by Spanish and French researchers evidences the possibility of gene flow from cultivated to wild material, possibility which deserves great attention if we consider the feasibility to introduce transgenic varieties of lucerne in open fields.

Evaluation of lucerne germplasm for agronomical and physiological traits has also been conducted in Spain for the Tierra de Campos ecotype (Fombellida, 2001), Greece (Vaitsis, 1999), Czech and Slovak Republics (Drobna *et al.*, 1999), Italy (Piano *et al.*, 1996; Torricelli *et al.*, 2003), France (Crochemore *et al.*, 1998) and Bulgaria (Keretikova *et al.*, 2003). An overview of the current state of lucerne genetic resources in Europe has been recently presented by Meglic *et al.* (2003). Last but not least, since the second half of the nineties more and more interest has been devoted to the characterization of lucerne genetic resources by means of molecular markers (Veronesi *et al.*, 1997).

## **B. GENETICS, BREEDING AND TRANSFORMATION**

### **B1. Genetics and cytogenetics**

A basic research area implemented in the last decade has been the one devoted to the analysis of reproductive mutants with possible interest for lucerne breeding (Barcaccia *et al.*, 1996; 2000; Tavoletti *et al.*, 2000). On the whole, the widespread occurrence of  $2n$  gametes (i.e. gametes with the somatic chromosome number) in the *Medicago sativa-coerulea-falcata* complex supports the concept that gene flow from diploid to tetraploid species occurs continuously in nature and plays a key role in lucerne evolution. Cytological investigation provided insights into the types of meiotic abnormalities responsible for the production of  $2n$  gametes. Alterations were defined as genetically equivalent to first (FDR) or second division restitution (SDR) mechanisms.

For breeding purposes, data have proven that  $2n$  gametes of the FDR type are more advantageous than those obtained by SDR for transferring parental heterozygosity and retaining epistatic interactions. The use of diploid meiotic mutants that produce  $2n$  gametes is now recognized as one of the most effective methods available for exploiting heterosis and introgressing wild germplasm traits into cultivated tetraploid lucerne via unilateral (USP) and bilateral sexual polyploidization (BSP) schemes. Furthermore, apomixis has the potential of cloning plants through seed and thus provides a unique opportunity for developing superior tetraploid varieties with permanent fixed heterosis and epistatic effects. A possible main goal in lucerne breeding could be the introduction of functional apomixis (i.e. apomeiosis and parthenogenesis) in cultivated lucerne stock. All this area has been recently reviewed by Barcaccia *et al.* (2003).

Molecular Markers (MMs) and mapping methods have been applied to lucerne by European researchers. After the pioneering paper of Kiss *et al.* (1993), a Restriction Fragment Length Polymorphism (RFLP) linkage map of a diploid lucerne meiotic mutant based on a  $F_1$  population has been obtained by Tavoletti *et al.* (1996a) who also performed a half tetrad analysis using RFLP to estimate the relative frequencies of  $2n$  gametes produced by mechanisms genetically equivalent to FDR or SDR (Tavoletti *et al.*, 1996b). At diploid level, Amplified Fragment Length Polymorphism (AFLP) fingerprinting in *Medicago spp.* has been developed and applied in linkage mapping by Barcaccia *et al.* (1999) while, more recently, the Sequence Specific Amplification Polymorphism (S-SAP) method, derived by the AFLP technique, has been utilized within the genus *Medicago* (with particular interest for the *M. sativa-falcata-coerulea* complex) to produce amplified fragments containing a retrotransposon LTR sequence at one end and a host restriction site at the other (Porceddu *et al.*, 2002). The technique appeared suitable for studying genetic diversity within, and relatedness between, lucerne species.

Considering that the cultivated forms of lucerne are autotetraploid ( $2n=4x=32$ ), population structure parameters commonly used for diploid species were re-examined and a specific population genetic software prepared (Ronfort *et al.*, 1999). MMs and mapping methods have been applied to  $4x$  materials using both AFLP dominant markers and microsatellite codominant markers (Simple Sequence Repeats, SSRs) (Julier *et al.*, 2003a). The genetic structure of lucerne populations and varieties makes the construction of such maps difficult. To reach the objective and to be able to detect Quantitative Trait Loci (QTLs) of breeding interest, specific mapping procedures for autotetraploids were applied using a  $F_1$  population produced from the cross of two heterozygous parental plants. The results were consistent with the hypothesis of a tetrasomic inheritance in lucerne. Furthermore, data coming from SSRs studies permitted to define that for most of the SSR loci Double Reduction (DR) was not significant. A further development in the area of estimation of DR coefficient (an essential feature of tetrasomic inheritance) is still

underway. SSR markers have been also used to analyse the structure of genetic diversity among and within lucerne varieties (Flajoulot *et al.*, 2005).

In order to clarify the domestication history of lucerne, Muller and co-authors (Muller *et al.*, 2001) applied a phylogeographical approach based on a characterization for mitochondrial DNA variation through RFLP of samples of natural and cultivated populations. In the wild pool from the presumed centre of origin (Near East to central Asia), diversity was high but the absence of geographic differentiation hinders a more precise location for domestication. Within cultivated lucerne, genetic differentiation associated with a geographic structure strongly suggests the existence of at least two independent routes for lucerne dissemination (East and West) from its centre of origin. The same authors studied DNA sequence diversity of a nuclear gene in wild and cultivated *M. sativa-falcata-coerulea* accessions concluding that a population bottleneck could be associated to lucerne domestication (Muller *et al.*, 2002). Cytological studies of the nucleolus organizing regions in the *M. sativa-coerulea-falcata* complex showed that the number of active Nucleolus Organizing Regions (NORs) in *M. sativa* is twice the number found in *M. coerulea* and in *M. falcata*. Consequently, if *M. sativa* originated from sexual hybridisation of  $2n$  gametes involving one or both diploid species, no major reorganization or loss of structural or functional rDNA loci occurred (Calderini *et al.*, 1996).

## **B2. Breeding, variety development, evaluation and identification**

The theoretical problems of lucerne breeding related to autotetraploidy have been carefully analysed in Europe a long time ago by Demarly, Gallais and Rotili (Demarly 1963; Gallais, 1977; Rotili 1976, 1979); nothing really new appeared in this area in the last decade. Mass, phenotypic, recurrent selection and synthetic variety development schemes are still the basis of variety development while the first so-called “chance hybrids” or “free hybrids” are entering the commercial seed market.

Considering new targets of breeding efforts for lucerne, the most important one, at least for Southern European countries, appears to be grazing tolerance. Traditional lucerne varieties generally present lower persistence under grazing management relative to mowing while in the last two decades lucerne grazing has aroused new interest due to the need for extensive livestock systems either to reduce the environmental risks related to intensive animal husbandry or to decrease the cost of production as a way to maintain competitiveness of livestock enterprises. On the basis of these considerations, Spanish, French and Italian research teams started selection programs for grazing tolerant lucerne (Delgado Enguita, 1989, Charrier *et al.*, 1993; Piano *et al.*, 1996). Some cultivars with a better survival under grazing were released such as Coussouls and Luzelle in France. Research is still in progress (Pecetti and Piano, 2005) and there is a hope that, in the

near future, European lucerne varieties specifically adapted to grazing will enter the seed market.

Other European selection targets appear to be improved seed yield (Svirskis, 1997; Huyghe *et al.*, 1999), DMY (Torricelli *et al.*, 2004), resistance to pea aphid (Bournoville *et al.*, 2002), persistence under frequent cutting regimes (Nagy, 2003), increased root size (Chloupek *et al.*, 1999), salt tolerance (Hefny and Doliski, 1999) and tolerance to soil acidity and aluminium toxicity (Hauptvogel, 1999).

DMY still remains the most important breeding target for lucerne and therefore it deserves a particular attention; if we look at data coming from Europe, in the last few years it is evident that DMY increases with respect to old varieties and local ecotypes have been very limited, being not more than 5% (Lloveras *et al.*, 1998; Kerticova and Scotti, 1999; Babinec *et al.*, 2003; Delgado *et al.*, 2003; C. Huyghe and F. Veronesi, unpublished data). In our opinion, the slow genetic gains are largely due to: (i) the very good adaptation of local populations; (ii) the wide genetic basis of the varieties; (iii) the small number of European research teams devoted to applied plant breeding; (iv) the inherent difficulties connected to the tetrasomic inheritance of the species. The presence of an important environmental influence on the varietal performances makes the investigation of variety x location interaction of special interest for lucerne, as indicated by the research work of Annichiarico and Piano (2005) based on Additive Main effects and Multiplicative Interaction (AMMI) analysis. The identification of morphological traits associated with general and specific adaptation patterns and possibly coupled in the long run with that on the relationship between adaptive traits and the genome level as revealed by MMs can improve the understanding of the variety x location interaction effects as well as assist plant breeding aimed at definite adaptation targets (Annichiarico, 1999).

Methodology of lucerne breeding with special interest to progress in molecular biology and to the use of MMs has been recently reviewed by Julier *et al.* (2003b) and by Veronesi *et al.* (2003). Thanks to the proximity to the model species *Medicago truncatula*, genomic databases, knowledge and tools have been expanded and theoretical developments made for the obtainment of linkage maps for an autotetraploid species such as lucerne. As a result, QTLs are located for an increasing number of traits and candidate genes will be shortly identified. In particular, the use of MMs in lucerne breeding can be characterized under four categories: (i) germplasm characterization and management, (ii) genetic linkage mapping, (iii) gene targeting and marker assisted selection, (iv) heterosis and inbreeding. MMs associated to useful traits have been already found (Scotti *et al.*, 2000; Julier *et al.*, 2002) even if, in spite of a quite large number of studies devoted to this area, no lucerne variety developed using marker technology has been released to date. However, MMs and DNA sequencing technologies evolve rapidly and we hope that these technologies will become available in the near future to lucerne breeders.

According to the opinion of some researchers, MMs could also be of interest for variety identification (Smolikova and Nedelnik, 1999; Labombarda *et al.*, 2000), even if the wide genetic variability typical of lucerne makes quite difficult to identify private bands useful for a real control of the commercial seed market.

### B3. Genetic transformation

In the last few years, research involving lucerne genetic transformation has been carried out by different European public research teams, predominantly in Belgium, France and Italy. According to Veronesi and Rosellini (2000), transformation efforts in Europe can be summarized as follows: (i) improved protein quality through the expression of seed storage proteins rich in sulfur amino acids such as  $\gamma$ -zein (Bellucci *et al.*, 2002); (ii) improved digestibility through the reduction of lignin content obtained with the inhibition of specific enzymes in the lignin biosynthesis pathway (Baucher *et al.*, 1999); (iii) male sterility obtained at Plant Genetic Systems, Gent, Belgium by transformation with a construct containing *Barnase* gene under the control of a tobacco anther tapetum specific promoter. The resulting transgenic male sterility has been characterized by Rosellini *et al.* (2001) who indicated that it should be possible to obtain good male sterile plants by backcrossing this trait into different genetic backgrounds; (iv) in an attempt to produce plants tolerant to soil acidity, lucerne has been transformed with a bacterial citrate synthase gene. Some transgenic plants showed a reduced aluminium absorption in root tips, a better root growth and a total DMY higher than controls in a greenhouse trial conducted with Al-toxic soil (D.Rosellini *et al.*, 2003); (v) basic research where lucerne genetic transformation is used as a tool for investigating the mode of action of genes involved in the plant-*Rhizobium* symbiotic interactions (Bauchrowitz *et al.*, 1994; Bauer *et al.*, 1996; Jimenez-Zurdo *et al.*, 2000); (vi) plastid transformation is presently attempted in several labs due to the potential of the plastome to produce high transgene expression levels in green tissue (Rosellini, personal communication) and to other advantages of plastome transformation over genetic engineering of the nucleus such as site specific integration.

## C. FORAGE QUALITY

Lucerne contains proteins which are rapidly degraded in the rumen; inducing a poor dietary efficiency, risk of bloat, and nitrogen loss detrimental to the environment. For these reasons, Julier and Colleagues have been active in the investigation of variation in ruminal protein degradability in lucerne (Julier *et al.*, 2003c). As a matter of fact, up to now the range of genetic variation within cultivated lucerne for *in situ* crude protein (CP) degradation appears to be narrow. Similar results have been obtained in a practical breeding program by Torricelli *et al.* (2001) who did not find a large variability for CP among lucerne plants. Even if these authors evidenced the absence of a negative correlation of

statistical significance between DMY and CP, they concluded that, from a practical point of view, too many single plants required to be analysed to apply selection differentials for CP within lucerne populations and to start a specific breeding program. It seems worthwhile paying more attention to the variability for CP among lucerne populations. The presence of an interesting genetic variation for quality components within the lucerne complex has been evidenced by Pecetti and colleagues in their research project aimed at developing grazing type lucerne varieties (Pecetti *et al.*, 2001).

Feeding value is an important trait for lucerne, which is characterized by high protein content but moderate energy value. Several studies have been performed to analyse the possibilities of genetic progress. In addition to laboratory measurements (enzymatic digestibility and fibre content, neutral detergent fibre (NDF) acid detergent fibre (ADF) acid detergent lignin (ADL), tools have been developed using near infrared spectroscopy (NIRS), making it possible to measure the feeding value for breeding purposes (Andueza *et al.*, 2001; Odoardi *et al.*, 2001). Large genetic variation was evidenced among varieties and among individual plants, and the inheritance of feeding value proved to be mainly additive (Julier *et al.*, 2003d).

On the basis of the above reported findings, new selection criteria have been added in lucerne breeding programmes, namely, trying to create more digestible varieties with high agronomic performances. The target is not a simple one, due to the negative genetic correlation evidenced between digestibility and forage yield (Julier *et al.*, 2003d). Molecular mapping is underway with the objective of identifying MMs associated with growth and digestibility traits to obtain a better understanding of the relationships among these traits and to produce tool kits useful for lucerne breeders.

Data coming from the dehydration industry seems to indicate that, at least for the moment, the choice of varieties is important for DMY while small differences in quality among varieties lead to the conclusion that agronomic technique, harvest frequency and weed control are the major factors influencing the quality of the dehydrated product (Corsi *et al.*, 2001). As a consequence, it should be clarified if breeding approaches for quality could be sustained by private breeders in the absence of a clear economic return in terms of higher market prices.

## CONCLUSIONS

The European experience in lucerne germplasm collection, conservation and evaluation shows the importance of this topic for the development of varieties adapted to grazing and resistant to environmental constraints. The use of MMs increases the possibility of studying the effects of gene flow from cultivated to wild materials. MMs and mapping methods have been applied to lucerne by European researchers. Thanks to the proximity



to the model species *Medicago truncatula*, genomic databases, knowledge and tools have been recently expanded and theoretical developments made for the obtainment of linkage maps for an autotetraploid such as lucerne. As a result, QTLs have been located for an increasing number of traits and candidate genes identified. Reproductive mutants may have impact on lucerne breeding; a main goal could be the introduction of functional apomixis (i.e. apomeiosis and parthenogenesis) in cultivated lucerne stocks.

Considering new targets for lucerne breeding, one of the most important appears to be grazing tolerance. Some cultivars with a better survival under grazing have been already released and there is a hope than, in the near future, new European lucerne varieties specifically adapted to grazing will enter the seed market. Other European selection targets appear to be improved seed yield, insect resistance, persistence under frequent cutting regimes, increased root size, salt tolerance and tolerance to soil acidity and aluminium toxicity. DMY still remains the most important breeding target for lucerne in Europe but it is evident that, in the last decade, increases with respect to old varieties and local ecotypes have been very limited, being on average 5%. Out of the several reasons which can be adducted for these slow genetic gains, the low number of research teams actually active in lucerne breeding in Europe should be mentioned.

In the last few years, research involving genetic transformation has been carried out by different teams with interesting results but at the moment the situation in Europe is very unfavourable to the release of GM crops. Lucerne has high risks of transgene dissemination due to its perenniality, outcrossing behaviour and long distance of pollen dispersal. There is also the presence of compatible materials such as local landraces and wild populations, some of them with a strategic importance for the maintenance of lucerne germplasm.

A new selection criterion, digestibility, has been added in the last few years to lucerne breeding programmes trying to create more digestible varieties with high agronomic performances. The target is not a simple one, due to the negative genetic correlation between digestibility and forage yield but molecular mapping is under study with the objective of identifying MMs associated to growth and digestibility traits useful for practical breeding.

On the whole, it looks like lucerne research in Europe reached a good scientific standard in the last decade but is carried out by few public research teams and is prevalently devoted to the analysis of basic problems; as a consequence, a bigger economic effort and a coordination between basic research and applied plant breeding are required priorities.

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## MEJORA GENÉTICA DE LA ALFALFA EN EUROPA: RESULTADOS Y ESTRATEGIAS DE INVESTIGACIÓN PARA FUTUROS DESARROLLOS

### RESUMEN

Se analizan los esfuerzos hechos en Europa desde el año 1995 sobre la mejora genética de la alfalfa en tres aspectos relevantes: (i) conservación y evaluación de colecciones de germoplasma; (ii) genética, mejora genética y transformaciones genéticas; (iii) calidad forrajera. En cuanto a recursos fitogenéticos europeos, se ha puesto especial énfasis en la búsqueda de material que pueda añadir nuevas características a la alfalfa cultivada, como la tolerancia al pastoreo y la resistencia a diferentes estreses. Los resultados sobre genética y mejora genética han sido más importantes en el área de las modernas biotecnologías y de la investigación básica que en la de creación de nuevas variedades. Las variedades más recientes se caracterizan por una producción forrajera media superior solamente en un 5% a la de las viejas variedades y ecotipos adaptados. Se analizan las razones de este escaso progreso. La tecnología sobre material transgénico está dando resultados muy interesantes, pero la actitud negativa de la opinión pública europea hacia las plantas genéticamente modificadas, y la posibilidad de transferencia de genes de plantas modificadas a variedades cultivadas, o a plantas silvestres, hacen difícil la hipótesis de poner en el mercado variedades de alfalfa genéticamente modificadas en el futuro inmediato. En los últimos años se ha observado un progreso en la creación de variedades más digestibles con alto valor agronómico.

**Palabras clave:** Germoplasma, transformación genética, características seleccionadas.