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Methodology of Alfalfa Breeding: A Review of Recent Achievements

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Abstract: In this paper, we review achievements recently reported in methodology of alfalfa breeding, with a focus on breeding criteria, mainly for forage quality and resistance to stress, and methodology, with a special concern to characterisation and management of genetic resources. A major part of the paper is dedicated to progresses in molecular biology. Thanks to the vicinity to the model species *Medicago truncatula*, genomic databases, knowledge and tools were developed. In the same time, theoretical developments were made for development of linkage maps for autotetraploid species. As a result, QTLs are located for an increasing number of traits. Candidate genes will be shortly identified. The possible applications of these advances are discussed, especially as they could provide new insights into the functioning of alfalfa canopies, that are complex because of the genetic diversity and of the complex physical structure. Recent achievements in transgenesis are also described.

Keywords: alfalfa; breeding; criterion; methodology; molecular biology; QTL; transgenesis

In this paper, we will review the recent achievements in methodology of alfalfa breeding. Knowledge on selection criteria was built up for several traits of agronomic importance for alfalfa. Genetic variation available with the species complex has been extensively described and provided an insight into the evolution of the species. But the major achievements are clearly in molecular biology. The technology offers the possibility to describe the complex alfalfa genome and to identify the areas involved into most agronomic traits. It also offers prospects of renovating our approach of alfalfa breeding.

Breeding criteria

It is not our objective to cover all the traits that are of interest in alfalfa breeding. We will only focus on some traits for which, to our mind, interesting break-throughs were observed since the last *Medicago* EUCARPIA Meeting.

Among the traits of major agronomic importance, those related to forage quality deserved major investments over the last decade. Forage digestibility has been regarded as a limiting factor of alfalfa when it has to be used in the diet of animals with high performance, such as diary cows. Julier *et al*. (2000) showed the importance of the within and among-cultivar genetic variation for this trait, this variation being exploitable in breeding as demonstrated by the successful divergent selection (Table 1) (Julier *et al*. 2003). Guines *et al*. (2003) showed that variation in forage digestibility was related to variation in stem morphology, the most digestible material showing higher proportion of medullar and cortical parenchymas. Sheaffer *et al*. (1999) analysed the genotype × environment interaction for alfalfa quality. They showed that harvests in the seeding year were not sufficient to predict the quality, measured through either NDF or ADF contents. They also recommended to use checks of low, mid and high quality, those checks being chosen to their stability against environmental variation. Changes in the kinetics of dry matter degradation through reduction of initial rate of degradation lead to the development of a bloat-reduced alfalfa cultivar (Coulman *et al*. 2000).

Table 1. Characteristics of three pairs of polycross coming from one and two cycles of divergent selection for digestibility (A+ vs. A-, B+ vs. B-, C+ vs. C-), compared to three control cultivars. NDF content, biomass and protein content are means over 5 cuts in 2 years for the material coming from the first cycle of selection, and over 2 cuts in one year for the material coming from the second cycle of selection. Lodging was scored from 1 (no lodging) to 5 (severe lodging)

Cultivar	Cycle I				Cycle II			
	NDF (%)	Biomass (g DM/plot)	Protein (% DM)	Lodging[1]	NDF (%)	DM yield (t/ha)	Protein (% DM)	Lodging[1]
Polycross A+	44.9 cd	413 a	14.8 b	3.3 ab	40.6 b	3.6 a	17.2 a	3.3 ab
Polycross A-	46.4 bc	411 a	14.1 c	2.0 abc	45.6 a	3.6 a	15.3 ab	3.5 ab
Polycross B+	43.9 d	391 a	15.5 a	2.8 abc	42.2 ab	3.5 a	17.5 a	3.7 ab
Polycross B-	45.8 bc	362 a	13.9 c	1.8 bc	44.3 ab	3.8 a	15.6 ab	3.0 abc
Polycross C+	44.1 d	386 a	15.4 a	2.3 abc	40.3 b	3.3 a	17.5 a	1.3 d
Polycross C-	47.5 a	382 a	13.1 d	1.3 c	45.4 a	3.8 a	14.7 b	1.8 cd
Check	45.8 bc	384 a	14.1 c	1.5 c	46.3 a	3.5 a	15.4 ab	2.5 bcd
Luisante	42.6 e	289 c	14.8 b	3.5 a	42.0 ab	3.4 a	17.2 a	4.3 a
Natsuwakaba	45.8 bc	326 b	13.9 c	2.8 abc	44.1 ab	3.3 a	15.8 ab	3.5 ab

For each trait, values followed by the same letter do not different significantly at $P = 0.05$

[1]scored on a single harvest

cv. Europe for cycle I, cv. Mercedes for cycle II

Protein degradation deserved some new investigations. Tremblay *et al*. (2000) showed little significant genetic variation in ruminal undegradable protein among a set of 27 cultivars on average over four harvests. Julier *et al*. (2003) found no variation among 15 alfalfa cultivars for protein degradation kinetics. When investigating both stem and leaves, Tremblay *et al*. (2003) showed that there was a significant variation in the proportion of ruminal undegradable protein in the leaves and that leaf proportion (or leaf to stem ratio) is likely to play a major role in determining alfalfa plant ruminal undegradable protein concentration. No data is available on the range of variation available within population or variety for those traits.

Improving grazing tolerance of alfalfa would be a major achievement but progresses are slow and difficult. Investigation of the physiological bases of tolerance to grazing was pursued. Two major orientations were explored. The first one is oriented towards structure of root system and especially the prospects offered by the creeping plants. Pecetti and Piano (2002) showed that genetic penetrance of creeping-rootedness in clonal progenies of alfalfa was never complete. When exploring the biochemical bases, the same group highlighted the high concentrations of crown carbohydrates and root and crown-soluble proteins of the plants expected to be grazing tolerant and characterized by prostrate, rhizomatous habit and long dormancy (Fornasier *et al*. 2003). Perez and Dall'Agnol (pers. commun.) investigated a new issue related to grazing tolerance. They investigated the possibility for grazing tolerance to be associated with the structure of the stem bud population. Indeed, after harvest or grazing this population of buds is strongly modified and reduced. Those genotypes with the highest tolerance to grazing are those which show many buds below the grazing height. This morphogenetical issue would deserve more investigation in the future.

Winter survival was of ten considered as a response to frost and low temperatures. Interesting contributions were made on the physiological aspects related to winter survival and especially root physiology. These contributions clearly showed relationship between winter survival and plant physiology, especially accumulation of nitrogen components in the roots during the preceding fall as a response to harvesting schedule and weather

conditions (Dhont *et al*. 2003). Furthermore, Cunningham *et al*. (2003), in monitoring root expression of galactinol synthase gene, demonstrated the role of raffinose family oligosaccharides in the genetic variation observed for winter survival. Haagenson *et al*. (2003) also stressed out role of cold-acclimation responsive (car) genes.

Breeding methodology

In this section related to breeding methodology, we will mainly focus on two issues. The first one is related to genetic diversity available within the complex of species and the second one to the prospect of exploiting the diversity through a maximisation of heterosis.

An appropriate knowledge of genetic resources and structuration is a pre-requisite of its preservation and of its optimum use in breeding.

Few papers assessing alfalfa genetic diversity were published recently. The survey run by Musial *et al*. (2002) using molecular markers on the genetic material used in Australia confirmed a wide range of variation within-cultivars and a low level of differentiation among cultivars. The results also indicated that *Medicago falcata* has not been widely used in Australian breeding programs and could offer a means of introducing genetic diversity into the alfalfa gene pool.

Even if most of the alfalfa genetic diversity is preserved in *ex situ* collection, it is of a crucial importance to analyse structure of wild populations when they exist and their exchanges of genes with the cultivated populations. A remarkable work was run by the group of Prospéri in Montpellier (France). They described the differentiation between natural (Mielga types) and cultivated populations of alfalfa in Spain. Using morphological traits, allozymes and molecular markers, gene flow from crop to wild was demonstrated but it was also evidenced that in some populations of Southern Spain natural selection may oppose gene flow to establish agronomic traits from cultivars into the natural introgressed populations (Jenczewski 1999a, b). Furthermore, using mitochondrial DNA, it was shown that gene flow occurred through seed exchanges from crop to the wild but also from wild type into traditional cultivated populations (Muller *et al*. 2001). This clearly points out the need to preserve these wild populations with adequate preservation programs.

The structure of the genetic diversity preserved in the *ex situ* collection, especially the very broad within-population variation, and the cost of this preservation should lead us to define the optimum preservation procedure. It could be envisaged to build up gene pool gathering material with similar agronomic traits. It could also be envisaged to carry out a dynamic management of this diversity to facilitate emergence of gene pool with high agronomic value.

The very broad genetic basis of most cultivars and reproductive biology of the species were regarded as the causes of the slow genetic progresses observed in alfalfa over the last decades (Riday & Brummer 2002). Brummer's group in the USA investigated the possibility of using *M. falcata* for maximising heterosis. In a spaced plant design, a significant heterosis (up to +18%) was observed in *sativa* × *falcata* crosses for forage yield (Figure 1) while no heterosis was detected for *sativa* × *sativa* crosses (Riday & Brummer 2002). However, this feature has to be confirmed under dense stands conditions where one of the features explaining heterosis (higher number of stems) is likely to be attenuated. To be exploitable, heterosis has to be predicted. When genetic distance was based upon neutral molecular markers (AFLP), it did not correlate with specific combining ability (Riday *et al*. 2003). Conversely, distance based upon morphological and forage quality traits correlated significantly with heterosis.

Molecular biology

The number of studies made on alfalfa using molecular biology is very low, because of the complexity of the genetics of this species. Mainly, the autotetraploidy is a limitation against the use of molecular markers, mapping, identification of QTLs. However, the recent molecular studies developed on the model species *M. truncatula* (Barker *et al*. 1990; Cook 1999) and the phylogenetic proximity to alfalfa offer prospects in starting programs in cultivated tetraploid alfalfa. Several points are stressed here.

Transfer of SSR markers from *M. truncatula* to alfalfa

Many SSR markers were developed in France and the USA, mainly from EST databases but also with enriched-banks (Huguet, unpubl.; Diwan *et al*.

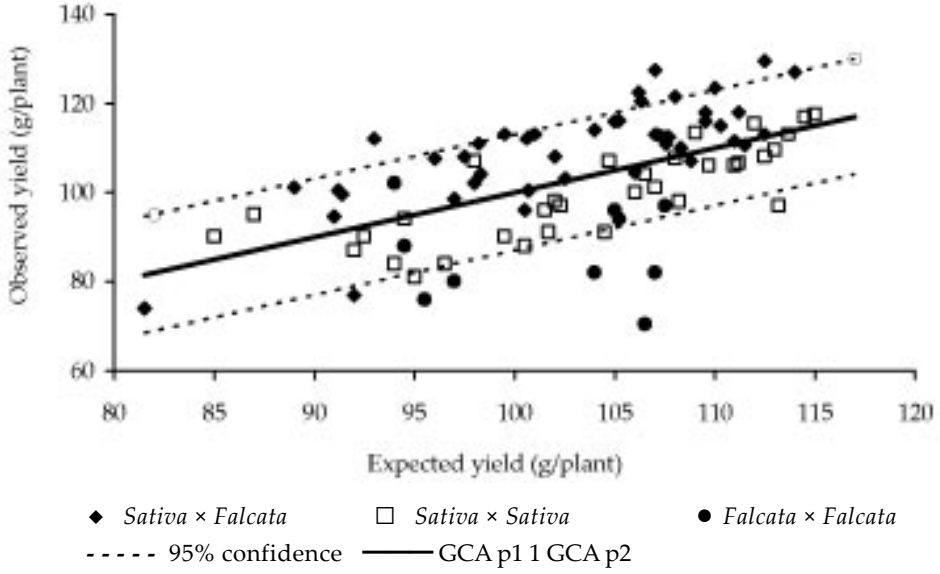


Figure 1. Observed versus expected experimental mean alfalfa dry matter yield (g/plant) across two Iowa locations and 5 harvests for alfalfa crosses (from Riday & Brummer 2002)

1997, 2000; Baquerrizo-Audiot *et al*. 2001, 2003). Attempts were made to use them in alfalfa (Julier *et al*. 2003; Campbell *et al*. 2003). About 70 to 80% of them amplify bands in alfalfa. About 50% reveale polymorphism when studying 2 heterozygous plants (Julier *et al*. submitted). The number of codominant markers available for genetic studies in alfalfa is now very high.

Genetic maps of tetraploid alfalfa

Fully saturated maps were obtained in diploid *M. sativa* (Kaló *et al*. 2000) and in *M. truncatula* (Thoquet *et al*. 2002). In both cases, the species are wild, so QTL research for agronomic traits is difficult or impossible. In tetraploid alfalfa, unsaturated maps were developed using RAPD (Yu & Pauls 1993), RFLP (Brouwer & Osborn 1999) or SSR markers (Diwan *et al*. 2000). In these maps, only simplex markers (marker present as a single dose in one parent, absent in the other – or SDRF for Single Dose Restriction Fragment, Wu *et al*. 1992) were used to calculate the recombination rates.

Theories were recently developed on mapping in autotetraploid species (Hackett *et al*. 1998; Ripol *et al*. 1999; Luo *et al*. 2000, 2001; Wu *et al*. 2001). These new theories are much more powerful than the previous methods. They make it possible to use all information provided by codominant markers, in which some alleles segregate as simplex, but others can segregate in duplex (double dose in one parent), or double simplex (single dose in both parents) or other. Hackett and Luo (2002) developed a software, TetraploidMap, available on Internet, to carry the calculations for mapping in autotetraploid species, using F_1 mapping populations.

Three groups are presently working on alfalfa mapping over the world: INRA at Lusignan in France (Julier *et al.* submitted), Noble Foundation at Ardmore in the USA (Sledge *et al*. 2003) and Iowa State University at Ames in the USA (Robins *et al*. 2003). Their objective is to develop genetic maps with a large number of SSR markers, because they are polymorphic, easy to handle and portable. The first group works with a mapping population made by the cross of plants coming from cultivated French varieties, although the other two work on *M. sativa* × *M. falcata* crosses.

At Lusignan, the genetic maps of both parents were based on SSR and AFLP markers. For each parent, 8 groups of 4 homologous chromosomes were obtained. The maps covered about 2900 cM, nearly 4 times the size of the haploid genome of diploid alfalfa that reached 754 cM (Kaló *et al*. 2000). The inheritance of codominant SSR markers showed that alfalfa is clearly an autotetraploid species, with no trend to disomic inheritance of markers. However,

Ma *et al*. (2002) found a preferential pairing in a *Sativa* × *Falcata* cross. The rate of double-reduction, that could arise from tetravalent formation at meiosis, was significant for some markers, but is probably related to segregation distorsion. A combined map, only composed of SSR markers was built. It covers 80% of the genome (Julier *et al*. submitted).

Colinearity of *M. truncatula* and alfalfa genomes

Comparison of genetic maps of *M. truncatula* and diploid alfalfa (Huguet, Kiss – pers. commun.) indicates a high level of synteny between both species. The alignement of *M. truncatula* and tetraploid alfalfa maps, based on common SSR markers, confirms these findings (Julier *et al*. submitted).

QTL identification

QTL research is much more easy and efficient in a diploid than in an autotetraploid species. But it is important to search for QTL in the cultivated species, and even in the genetic background used for breeding. However, another way could be to identify QTL in a diploid model species, and to validate the QTLs or possibly the genes in the cultivated species.

In tetraploid alfalfa, Brouwer *et al*. (2000) found QTL for frost resistance. Robins *et al*. (2003) mapped QTLs for forage yield. In our group, we identified QTLs for aerial morphogenesis (stem elongation rate, stem height, flowering date, number of stems per plant), that explained up to 60% of the phenotypic variation (Julier unpubl.). In parallel, QTLs for aerial morphogenesis were found in a RILs population of *M. truncatula* (Huguet & Julier unpubl.), with one to three major QTLs for each trait.

Considering the huge efforts required to build mapping populations and to map them, it would be of a major interest for the international scientific community to build up large databases on a few of such mapping populations, collecting as many traits as possible on the same populations. Besides questions on the availability of the information, a major limitation is the F1 status of the individuals of the mapping populations. This implies that they have to be multiplied through cutting and shipped as fresh and living material. It could also be envisaged to derive inbred lines from each of the individuals, these lines being maintained as seed.

For traits known to be oligogenic, bulk segregant analysis (BSA) was successfully used to locate QTL. Obert *et al*. (2000) identified four AFLP fragments associated with resistance to *Peronospora trifoliorum*.

Research of candidate genes

Recent studies on various species are leading to the knowledge of the sequence, promotors, regulation of genes involved in numerous traits. Some of these traits are related to the agronomic value in alfalfa: flowering date, vegetative growth, lignification… When these genes are described in *Arabidopsis thaliana*, or better in *M. truncatula*, their conservation across species and genera offers the possibility to test if they are involved in the variation for the same traits in alfalfa. Several methods can be used to analyse the effect of one gene on the phenotypic variation.

In the first method, after defining molecular markers in the gene (CAPS, STS or SNP), if variation for the markers is available, the gene can be mapped in a genetic linkage map, and the colocation of the gene with a QTL would be an indication.

The second method is to analyse the phenotype of banks of mutants. Such banks were not produced in alfalfa, but in *M. truncatula*. Indeed, a highly efficient transformation method was developed in *M. truncatula*, and programs of transposon-tagging were started. A T-DNA bank of mutants were thus produced (Scholte *et al*. 2002), and more recently, a TNT1 bank is on the way (D'Erfurth *et al*. 2003). The work thus consists in phenotyping a large number of mutants, and to analyse more precisely the mutants carrying peculiar phenotypes. The isolation of the genes that have been modified is the first step to study their function and regulation. The genes can then be mapped. Similarly, tilling populations are being produced in France, UK and the USA in *M. truncatula*, as in *A. thaliana* (Till *et al*. 2003; Greene *et al.* 2003).

The third method is still emerging in plant biology, and consists in association study. The theory, well described in human genetics, is based on the analysis of populations, in which a variability for a phenotypic trait is known. The genotype of each individual is described for each candidate gene, and a statistical analysis is handled to relate ge-

netic variation at one gene to phenotypic variation. Even if theoretically any marker can be used, the linkage desequilibrium, especially in allogamous species such as alfalfa, is so small that the marker can have a detectable effect on the phenotype only if it is located within the gene or very close to it (Flint-Garcia *et al*. 2003). Background is lacking on plants to analyse the efficiency of this method (see Thornsberry *et al*. 2001 on maize).

A last possible method is to generate EST banks using the suppressive subtractive hybridisation method. It is mainly used for interaction between plants and pathogens or symbiots but could possibly be used in a wider range of conditions. Such banks are enriched in genes directly involved into plant reaction to a peculiar situation. Genes must then be screened for their possible status of candidate gene. Wulf *et al*. (2003) used this methodology for analysing the genes involved into the interaction between *M. truncatua* and *mycorrhiza*. This approach is presently developed in Toulouse, France for symbiotic fixation by P. Gamas and in Angers for seed germination by A. Limani.

Possible applications of molecular biology in alfalfa

A first practical application is the analysis and management of genetic resources. The codominant SSR markers give a more detailed information than dominant markers such as AFLPs, and are much more numerous than the previously used isozymes or the RFLPs. Banks of alfalfa genetic resources could use this information to make decision on the maintainance of the populations and to analyse possible genetic variation occurring during the multiplication generations as a consequence of genetic drift. For a breeder, it could be a useful tool to assess the genetic diversity available in his breeding pools and its structure.

A second application is related to breeding. Even if QTLs could only be used in the cross in which they have been identified, we can expect that genes, when proved to be involved in agronomical traits, could be used to fix rapidly the favourable alleles in the populations. The way is probably long for quantitative traits, but marker assisted selection (MAS) for monogenic traits like disease resistance could become available shortly. In diploid species, it was shown that MAS was more efficient for traits with low heritability. In tetraploid species, in which the genotype is even more poorly estimated from the phenotype, the impact of MAS in breeding progress could be very valuable.

A third possible use would be the identification of variety. Because of the number of registered varieties and their complex genetic structure, distinction of varieties is becoming a critical (and expensive) issue in the registration procedure. This feature is true in most countries. Use of co-dominant markers, such as SSR would provide extra information alongside with slight difference for morphological or physiological traits to support a decision of distinction. Both mean allelic composition and panmictic equilibrium for molecular markers would be informative.

A fourth application would be to analyse the changes of the plant population after seeding. In the 1970's, Rotili and Zannone (1975) noticed that a large proportion of seedlings and plants died all along the life of the canopy. Indeed, the seedlings, and then the plants compete for light, water and mineral nutrition. The surviving plants are those that successfully compete. As alfalfa varieties are populations with a large genetic basis, the death of a large proportion of plants can induce a genetic shift. Rotili *et al*. (1985) stated that the genetic diversity within varieties increased between-plant competition, because some plants were more able than other to grow more in certain periods of the year. The situation is similar in alfalfa seed crops even if the sowing density of such crops is 10 times lower than for forage crops. Indeed we demonstrated that, even in absence of variation in seed production, the plant population quickly changed over the years of production (Table 2) and that, in Y2, 21% of the plants actually present, each carrying more than 4 stems, contributed 50% of the seed production (Table 3). If this strong contribution to yield is associated to specific traits or features, a quick drift in the genetic structure of the variety may be expected. Molecular markers are use-

Table 2. Effect of the age of seed crops on canopy structure in alfalfa at Lusignan (2000)

	No. plants/m	No. stems/m	No. inflorescences/m
Y0 (sowing year)	136	173	746
Y1	62	154	858
Y2	70	152	1022

Table 3. Contribution of the various plant types (number of stems per plant) to seed yield in Y0 and Y2 alfalfa seed crops

Stems/plant	Y0		Y2	
No fertile stems/plant	No. plants/m	% yield	No. plants/m	% yield
0	55	0	14	0
1	109	61	24	12
2	19	22	20	22
3	7	14	11	16
4	1	3	6	16
5			3	11
6			2	8
7			2	6
8			2	9

ful tools to analyse the genetic evolution in the canopies. Neutral markers, as are supposed to be SSRs, or non-neutral markers as candidate genes, can help in these studies even if they do provide different information. A better understanding of the genetic changes within a canopy would help in defining the optimum genetic basis of varieties. Is a broad-basis cultivar more suited to survive and produce in various environmental conditions and managements than a narrow-basis cultivar?

Transgenesis

This technique has been used in alfalfa for 30 years. Different types of genes were introduced in alfalfa, and can induce positive phenotypes.

Herbicide-resistant genotypes were developed, and varieties are under registration in the USA. Such transformed plants will probably be poorly accepted by the European consumers, because they do not give rise to important progress.

Other transgenic genotypes were recently produced and could be of interest for farmers. Transgenic alfalfa, modified for lignin pathway (Baucher *et al*. 1999; Guo *et al*. 2001a,b, 2002; Marita *et al.* 2003), showed improved dry matter digestibility and modification in lignin composition. Gruber *et al*. (2003) announced that a transgenic alfalfa expressing a precursor of condensed tannins in the leaves was obtained. It could be a way to reduce protein degradation in the rumen. Cloning of a gene coding for polyphenol oxidase gene (PPO) in red clover was made, and the transformation of alfalfa was obtained (Sullivan *et al*. 2003). PPO is responsible in red clover of the low proteolysis during ensiling.

Transgenic plants, possibly better adapted to the environment, were produced. Aluminium-tolerant plants were obtained (Tesfaye *et al.* 2001), as plant expressing a gene possibly responsible for frost resistance (McKersie *et al.* 1999, 2000). The effects of superoxide dismutase genes (SOD) on persistence and biomass production (Samis *et al.* 2002) and possibility on water stress tolerance (Rubio *et al.* 2002) was described. The resistance to pests or insects could also be introduced, when the genes leading to the resistance are known and identified. The expression of resveratrol synthase (Hipskind & Paiva 2000), or of isoflavone O-methyltransferase (He & Dixon 2000) both induced increased resistance of alfalfa to *Phoma medicaginis*.

Transgenesis could also lead to modification in the breeding methodology as Rosellini *et al*. (2001) reported the production of male sterile transgenic plants after transformation with a RNAase gene from *Bacillus amiloliquefaciens*.

Another objective would be to produce alfalfa expressing drugs, that could be extracted from the plants, and that would be safer than when produced or extracted from animals and cost-effective. Khoudi *et al*. (1999) described the production of monoclonal antibodies in transgenic alfalfa. A Canadian company (Medicago Inc.), located in Sainte-Foy (Québec), is developing transgenic

alfalfa for such medical purposes. Similarly, the possibility to produce a plastic biopolymer in alfalfa was described (Saruul *et al*. 2002).

Conclusion

As briefly shown in this paper, major changes happened in alfalfa breeding over the last few years.

Breeding criteria were finely tuned as a consequence of a better understanding of the physiological basis of the traits under breeding. A more comprehensive characterisation of the genetic diversity available in the species complex is becoming available and new prospects are offered for better exploiting this diversity.

But, without any contest, the most remarkable feature is the break through in molecular biology, mainly thanks to the major achievements on *Medicago truncatula* used as a model species for all legumes but also thanks to progresses in the genomics of and theoretical achievements on autotetraploid species. International collaborative actions are initiated and more could be done to better valorise the available mapping populations. It is now possible and under progresses to identify QTLs and candidate genes, opening possibilities for marker assisted selection. Tools are also available for monitoring and optimally use genetic variability. It will now be possible to monitor the fate of individual plants and the fate of peculiar alleles in a given canopy. Doing so, all concepts and models of population genetics may be applied to these cultivated swards. It will of course offer new prospects for breeding and above all completely renovate our concepts, approaches and methodologies of breeding this species.

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