REVIEW

Questions and Avenues for Lucerne Improvement

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Abstract: Six crucial questions for lucerne breeders are set up and discussed in relation to the available information. (i) Which width of adaptation? Genotype × location interaction is region-specific and may be wide enough to justify breeding for specific adaptation. Genotype × exploitation interaction requires contrasting plant types for mowing and intensive grazing. (ii) Can we breed very drought-tolerant varieties? One drought-tolerant landrace exhibited a drought-avoidance, water-conservation strategy based on limited root development, while large root featured material adapted to favourable environments and/or frequent mowing. (iii) Which selection scheme and variety type? Many schemes were proposed for synthetic varieties, but empirical or theoretical comparisons were limited in number and inference space. Non-additive genetic variation may be exploited by free hybrids (semi-hybrids) through procedures varying in complexity, possibly assisted by marker evaluation. Previous selection of exotic germplasm for adaptation is essential. (iv) How to improve the forage quality? Selection for modified stem morphology (increased internode number, decreased internode length) proved effective. Combined selection for forage yield and leaf/stem ratio seems also feasible. (v) Which opportunities for marker-assisted selection? Linkage maps for lucerne are available but useful markers for forage yield may be site-specific. Bulk segregant analysis is promising in breeding for stress tolerance. (vi) How to exploit genomic information from *M. truncatula*? This model species can help in developing markers and locating genes which control metabolic pathways, such as saponin content and composition. Information from *M. truncatula* on marker-trait association for forage yield or tolerance to abiotic stresses may be little exploitable.

Keywords: drought tolerance; forage quality; genomics; grazing tolerance; heterosis; markers

Lucerne (*Medicago sativa* L.) is a major crop in temperate regions whose importance may further increase because of its contribution to sustainable agriculture and its productivity of feed proteins per unit area which is the greatest among forage or grain legumes (Huyghe 2003). This species has displayed low rates of genetic gain for forage yield compared with other crops, namely 0.2%–0.3% per year relative to about 2% for maize and 1% for white clover (Woodfield & Brummer 2001). Breeders have successfully improved lucerne tol-erance to various diseases, but little progress has been achieved as regards intrinsic yield potential or forage quality of the crop (Rotili *et al.* 1999a; Lamb *et al.* 2006). Various factors may account for this trend, such as autotetraploidy, high rate of non-additive genetic variance arising from gene interaction and high genotype × environment (GE) interaction, besides the outbreeding mating system and the perennial growth cycle. With-out any claim to be exhaustive, our objective in this paper was setting up some questions which

we feel as crucial for lucerne breeders, trying to anticipate some answers also on the basis of our research work.

Breeding for wide adaptation: how wide?

This question recalls that expressed by Cecca-relli (1989) in the general context of plant breeding. It relates to challenges and opportunities offered to breeders by GE interactions and, in particular, the pros and cons of selecting for specific adaptation to distinct pedoclimatic areas or crop management conditions (Annicchiarico 2002).

The variation in lucerne exploitation mode (mowing; grazing by different animals and intensity) represents a source of GE interaction. Specific adaptation to definite exploitations depending on the morphological type emerged in breeding of grazing-tolerant lucerne. Germplasm of the *M. sativa* complex was classed into four types depending on its morphology and vigour (Piano *et al.* 1996), and selections for each type were compared under different exploitation modes. Table 1 summarizes the results for two contrasting types, i.e.: (i) D1, which is very rhizomatous, with prostrate habit and great sideways-spreading ability; and (ii) D4, which is semi-erect with a conspicuous shoot proliferation from a broad crown. Continuous sheep grazing and mowing contrasted sharply for the cultivar response, and require distinct plant type and selection work (D4 type being preferable for hay + rotational grazing exploitation).

The extent of genotype × location (GL) interaction requires verification for the target region. If it is sizeable, it may be exploited by breeding for distinct subregions or minimized by breeding for wide adaptation. In the AMMI (Additive Main effects and Multiplicative Interaction; Gauch 1992)

modelling of a set of variety trials in northern Italy, the cross-over GL interaction for forage yield was large (Figure 1) and repeatable in time, was related to soil type and summer drought-stress level of the sites, and implied specific adaptation to the area where the cultivar or its genetic base originated (Annicchiarico 1992). Two geographically-defined, contrasting subregions emerged from the site classification for GL effects: (i) subregion A in the north-western Po valley, characterized by sandy-loam to loam soil and by limited drought stress mainly due to irrigated cropping; and (ii) subregion C in the south-eastern Po valley, tending to clay soil and to severe summer drought due to rainfed cropping and somewhat lower rainfall. Subregion B was intermediate between A and C geographically, for cultivar response and environmental characteristics. Varieties selected in Lodi, which is placed in subregion A, tended to be specifically-adapted to this subregion (e.g. Robot; Figure 1A), but the adoption of a second selection site placed in subregion C was prevented by its cost. Four artificial environments created in Lodi by the factorial combination of soil type (sandy-loam or silty-clay) and drought stress (almost nil or high) successfully reproduced the adaptive responses across agricultural environments of three reference varieties and confirmed 'no stress/sandy-loam soil' and 'stress/silty-clay soil' (representing the environments of subregions A and C, respectively) as the most-contrasting for cultivar adaptive response (Figure 1B), promoting the artificial environments as a cheaper alternative to two selection locations. There was a close relationship between drought tolerance of a set of lucerne farm landraces (of which a subset is reported in Figure 1B) and drought-stress level in their evolution environments (Annicchiarico & Piano 2005). The comparison of wide vs. specific breeding for the two subregions based on actual

Table 1. Persistence of two morphological types of lucerne under different exploitations

Exploitation	Sampling time	Persistence (%)		Source
		type D1	type D4	
Sheep continuous grazing	2nd year	87.5[a]	56.9[b]	Pecetti *et al.* (2008)
Cattle rotational grazing	4th year	55.0[a]	53.7[a]	Pecetti *et al.* (2007)
Horse rotational grazing	4th year	57.1[a]	51.5[a]	Pecetti *et al.* (2005)
Mowing (organic system)	3rd year	22.4[b]	36.1[a]	Annicchiarico and Pecetti (2008)

Row means with different letter differ at $P < 0.05$; see text for description of plant types
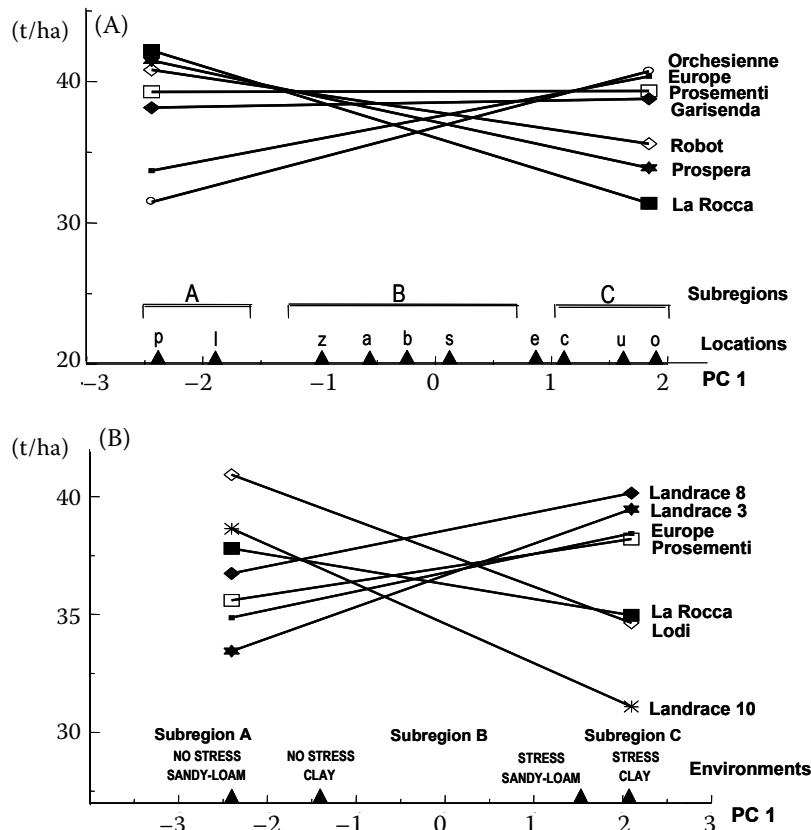
Figure 1. Nominal dry matter yields (i.e. modelled yields from which the environment main effect, irrelevant for entry ranking, has been removed) for: (i) seven varieties as a function of the score on the first genotype × location interaction principal component (PC) axis of 10 coded agricultural locations (A); (ii) four varieties and three farm landraces as a function of the score on the first genotype × environment interaction PC axis of four artificial environments (B); source: Annicchiarico (2002) (modified)

yield gains from phenotypic selection in the artificial environments indicated a distinct advantage of specific breeding (Annicchiarico 2007a). Specific adaptation was related to different optima of root biomass, leaflet size and winter growth (Annicchiarico 2007b). Large GL interaction effects are likely to occur in lucerne (or other forage crops) because of the long-standing, site-specific selection pressures that acted on landraces or natural populations and the large use of such material for variety selection.

While specific breeding may be envisaged for contrasting exploitation modes or geographical areas, there is little evidence that distinct lucerne varieties are required for organic farming following the high correlation for cultivar forage yield between conventional and organic growing conditions (Annicchiarico *et al.* 2007).

Another type of GE interaction which is relevant to lucerne breeders is genotype × plant density interaction, as selection has frequently been performed under spaced planting while targeting dense-planting environments. Several studies reported a low phenotypic correlation between these conditions, owing to the different plant traits associated with better response in each

condition (Rotili & Zannone 1975). Selecting under spaced planting implied a predicted loss of efficiency of 36% for forage yield and 19% for seed yield relative to selection under dense planting (Annicchiarico 2006a), supporting the adoption of relatively dense-planting procedures also for phenotypic selection of individual plants (Rotili *et al.* 1999a; Annicchiarico 2004).

Can we breed very drought-tolerant cultivars?

This issue, which actually belongs to the range of issues relative to adaptation targets, is specifically addressed because of its increasing importance in the context of climate changes (Fischer *et al.* 2002). Lucerne has a reputation as a fairly drought-tolerant crop (Sheaffer *et al.* 1988), but there is scant investigation of the genetic variation for drought tolerance and its underlying physiological mechanisms.

The EU-funded project "Improvement of native perennial forage plants for sustainability of Mediterranean farming systems" (PERMED) has contemplated the assessment of adaptation and drought tolerance for a number of lucerne populations across south-European and north-African

Table 2. Root weight (between 2 and 68 cm depth) prior to drought stress, and soil water consumed between the occurrences of mild and severe stress (based on soil humidity measures) for four lucerne cultivars

Cultivar	Origin	Adaptation	Root weight (g/plot)	Soil water consumed (%)
Mamuntanas	Sardinia	drought-stress sites	8.9	1.5
SARDI 10	Australia	wide	14.9	1.2
Prosementi	Northern Italy	moderately favourable sites	12.0	0.7
Demnat	Morocco	very favourable sites	16.4	0.5
LSD ($P < 0.05$)			3.4	0.6

agricultural environments, followed by the investigation of morphophysiological traits associated with drought tolerance in separate physiological studies. Some preliminary results relative to four cultivars of contrasting adaptation across agricultural sites are anticipated in Table 2. Root biomass and soil water consumed between the occurrence of mild and severe stress were estimated in metal containers (55 × 12 × 75 cm deep). The drought tolerance of the Sardinian landrace Mamuntanas was mainly due to a drought-avoidance, water-conservation strategy based on limited root development, which implies more water available in late, severe stressing periods. Limited root biomass already emerged as a feature of the material specifically adapted to water-limited Italian environments (Annicchiarico 2007b), probably because the large investment of photosynthate required for growth, function and maintenance of an extensive root does not repay the additional water uptake in rainfed Mediterranean environments (where spring and summer rainfalls hardly reach deep soil layers). Large root development featured the oasis landrace Demnat specifically adapted to favourable cropping environments (Table 2), in agreement with previous indications on the importance of this trait in such environments (Annicchiarico 2007b) and/or under frequent mowing (where greater storage of nitrogen reserves associated with the larger root contributes to better persistence and ability to withstand the severe intra-specific competition for light and nutrients: Avice *et al.* 1997).

Which selection scheme and variety type?

A number of selection schemes have been proposed for lucerne synthetic varieties, but empirical comparisons (summarized in Rumbaugh *et al.* 1988) have been rare and limited to just a few schemes, whereas theoretical comparisons (e.g. Busbice 1970; Casler & Brummer 2008) were hindered by the absence of reliable estimation for relevant genetic parameters. A large comparison of selection schemes, summarized in Figure 2, is on-going in Lodi. The base population included a large collection of landraces from northern Italy and a set of elite, locally-adapted varieties (Annicchiarico 2006b). A phenotypic selection stage, which is likely to be present in any breeding program prior to the adoption of a given selection scheme, was performed as described in Annicchiarico (2004). Cloning ability of genotypes (which is a prerequisite for clonal selection) usually proved sufficiently high. Selfing of genotypes (which is a prerequisite for all schemes contemplating S_1 material) produced sufficient S_1 seed in the vast majority of cases, whereas 33 out of 125 genotypes did not produce sufficient seed for evaluation of S_2 progenies. The efficiency of selection schemes will be compared on the ground of actual selection gain attained by the relevant experimental synthetics (which are currently being produced).

Lucerne may show large heterosis for forage yield mainly because of non-additive, complementary gene interactions between different alleles organized in 'linkats' (which can be wider in an autotetraploid species) (Bingham *et al.* 1994). Elite parent material maximizing the genetic diversity would be expected to also maximize heterosis and produce, thereby, higher-yielding synthetic varieties. However, synthetics which maximized the genetic diversity as assessed by molecular markers did not display a sizeable yield advantage over those minimizing this diversity (Kidwell *et al.* 1999), suggesting that marker-based diversity accounted poorly for, and was little related to, the diversity in genomic regions affecting forage yield.
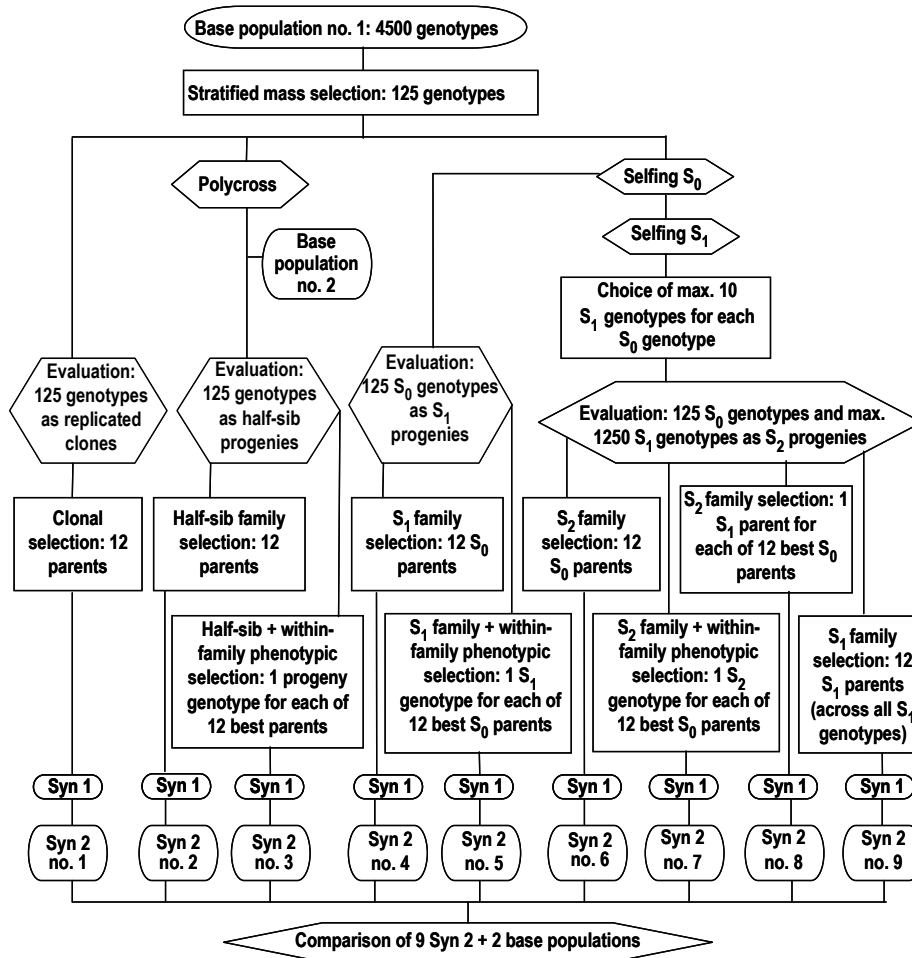
Figure 2. On-going comparison of nine lucerne selection schemes in Lodi

Also in other contexts, such as crosses between populations, marker-based genetic diversity may not be sufficient to predict heterosis, owing to the absence of linkage disequilibrium or to highly overlapping diversity between populations (Riday et al. 2003).

Hybrid varieties of lucerne, which are generated by crossing genetically-distant, relatively narrow-based parent populations through a patented male-sterility technique, offer a simple means of exploiting heterosis. The first hybrid varieties, which are expected to have at least 75% hybridity (as male-sterile and male-fertile parents are planted at a seed ratio of 4:1), have not shown yet a distinct yield advantage over traditional synthetic varieties (Riday et al. 2008) but their potential is expected to rise.

Another avenue for increasing heterosis is producing free-hybrids (implying no male-sterile parent) between genetically-distant, well-complementing populations which were previously subjected to separate selection (Rotili et al. 1996; Brummer 1999). An important part of genetic variation in lucerne is between subspecies (*falcata*; *sativa*) of the *M. sativa* complex. Riday and Brummer (2002, 2006) and Maureira et al. (2004) found a *sativa-falcata* heterotic pattern but remarked the obstacle represented by the lack of *falcata* germplasm with high agronomic value. Furthermore, the requirements for variety homogeneity of the EU variety legislation set a limit to the width of the genetic distance between putative heterotic populations, discouraging the use of interspecific *M. sativa* × *M. falcata* free-hybrids and suggesting to locate heterotic populations within geographically or morphophysiologically distant germplasm pools of *M. sativa* subsp. *sativa* and × *varia*. Besides, variation within the subspecies *sativa* may also offer opportunities for exploiting heterosis through the proper management of geographic diversity. Selfing may further contribute to widen the genetic diversity within this subspecies.

Non-dormant exotic *sativa* germplasm is a likely candidate to express heterotic pattern towards semi-dormant European breeding material, in which contacts with subsp. *falcata* have occurred more or less consistently. Oases in the Sahara desert can be an environment of choice for the survey of putatively 'pure' subsp. *sativa* because of the long history of lucerne cultivation, geographic isolation and agroecological specificities (e.g. soil and water salinity) hampering the introduction of non-adapted germplasm. The comparison between Egyptian farm landraces collected in the Siwa oasis, Egyptian cultivars and Italian varieties including Egyptian parent germplasm confirmed that Siwa landraces could be distinguished both at the bio-agronomic and molecular level (Carelli *et al.* 2009). They also displayed forage yield not significantly lower than that of the cultivars, although under testing conditions (unheated open greenhouse) preventing the combined effects of cold and soil moisture which are typical of winter field conditions. Their genetic distinctness supports the hypothesis that this germplasm could act as a heterotic pool towards European semi-dormant material. Besides, Siwa germplasm can provide useful bioagronomic traits, e.g. high stem elongation rate during regrowth, autumn growth and larger seed size, thereby complementing those of the Italian germplasm. Improved, partly inbred (S_2) families from Siwa germplasm were developed and are currently crossed in a diallelic scheme to improve S_2 families derived from Italian ecotypes.

The use of selfing characterizes the breeding methodology devised by Rotili in Lodi (Rotili *et al.* 1999a). Selfing can be useful for increasing the effectiveness of selection (Rotili 1976), homogenizing the plant material for physiological traits, concentrating the genetic structures (linkats) favourable to vigour and other quantitative traits and unmasking the genetic load, as well as increasing the effectiveness of exploring the genetic diversity. By selfing and selection, the different genetic structures of the parental populations can be split, analyzed and concentrated into distinct individuals and families, generating partly inbred (S_2) improved families with high genetic distinctness. Crossing such families originated from populations with contrasting geographic origin and/or physiological traits within subspecies *sativa* is likely to highlight positive and consistent combining abilities among populations for important agronomic traits (Rotili *et al.* 1999b).

Table 3. AMOVA analysis of 6 parental multiplied simple hybrids (2S$_2$Syn3) based on 67 SSR markers

Source of variation	df	Variance components	Percentage of variation
Among families	5	20.79	37.4***
Within families	98	34.79	62.6***

***$P < 0.0001$: probability of obtaining more extreme component estimates by chance alone

The best S_2-simple hybrids thus obtained can be maintained by polycross as 2S$_2$-synthetics and constitute an effective way for storing the genetic variation and providing a set of parents for the construction of double (or 4S$_2$-component) free-hybrids. An example of the genetic distinctness of the 2S$_2$-synthetics obtained by this method is shown in Table 3, where the genetic diversity is estimated by means of 67 SSR markers (Carelli *et al.*, unpublished results).

The exploitation of heterosis requires suitable variety models which can effectively capitalize the heterosis effects. To assess the importance of the variety model, we compared free-hybrids and synthetics built up with the same four S_2 components which maximized the genetic diversity (i.e. each component originated from a different parental population) (Figure 3). The four S_2 components were crossed in a diallelic scheme giving rise to six simple hybrids or, altogether, to the Syn1 generation of the synthetic variety. The simple hybrids were then either polycrossed for three generations to obtain the 4S$_2$-component synthetics (Syn4 generation) or separately multiplied until the 2S$_2$Syn3 generation and finally crossed to produce 15 4S$_2$-component free-hybrids or double hybrids (Figure 3). All crosses/multiplications were hand-made without flower emasculation. Selection for vigour within families was performed in each generation. The comparison of parental 2S$_2$Syn3 synthetics, double hybrids and the corresponding 4S$_2$Syn4 synthetics was carried out in a cold greenhouse for forage yield over 10 harvests for two independent diallelic sets (diallel A and B). The results indicated that (Figure 4): (i) the main source of variation among crosses was represented by specific combining ability (SCA); (ii) positive and significant high-parent (HP) heterosis was found in each diallel; (iii) double hybrids, on average, did not differ significantly from the corresponding synthetic, but the best double-hybrids outyielded the synthetic
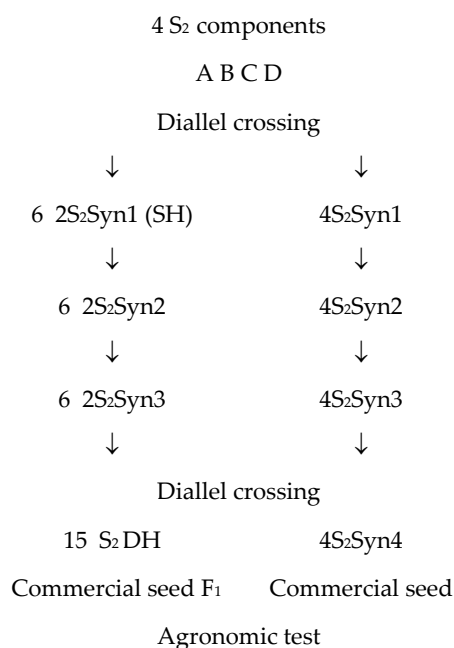
4 S$_2$ components

A B C D

Diallel crossing

$\downarrow$ $\qquad\qquad$ $\downarrow$

6 2S$_2$Syn1 (SH) $\qquad$ 4S$_2$Syn1

$\downarrow$ $\qquad\qquad$ $\downarrow$

6 2S$_2$Syn2 $\qquad\qquad$ 4S$_2$Syn2

$\downarrow$ $\qquad\qquad$ $\downarrow$

6 2S$_2$Syn3 $\qquad\qquad$ 4S$_2$Syn3

$\downarrow$ $\qquad\qquad$ $\downarrow$

Diallel crossing

15 S$_2$ DH $\qquad\qquad$ 4S$_2$Syn4

Commercial seed F$_1$ $\qquad$ Commercial seed

Agronomic test

Figure 3. Scheme of 4-component free-hybrids construction (SH – simple hybrid; DH – double hybrid)

by 10% and 25%, respectively, in the two diallel sets (Carelli *et al.* 2007; Scotti & Brummer 2009). The genetic diversity between parental 2S$_2$Syn3, estimated by means of SSR markers, was significantly correlated with hybrid performance and hybrid SCA percentage (Table 4). The prevailing role of SCA in double-hybrid responses is remarkable. Segovia-Lerma *et al.* (2004) in diallel analyses among the nine historically recognized lucerne germplasms, and Riday and Brummer (2002) in a diallel of nine elite *M. sativa* and five *M. falcata* genotypes, found a prevalence of general combining ability (GCA), while SCA effects were only detected in the hybridization of genetically distinct populations. Rotili and Zannone (1977) found a lower extent of SCA effects compared to GCA ones using the same 4-component hybrid model where, however, the 4 S$_2$ components originated just from two populations.

Table 4. Correlation between genetic distances of parents (2S$_2$Syn3) and performances of double hybrids

	r
Dissimilarity (1-Dice coefficient) *vs* DM yield	0.59*
Dissimilarity (1-Dice coefficient) *vs* HP-heterosis	0.70***
Dissimilarity (1-Dice coefficient) *vs* SCA	0.76***

*$P < 0.05$; ***$P < 0.001$

Selfing and selection in the S$_2$ component production, the genetic diversity of the S$_2$ components and their combining in the double hybrid model may account together for the high expression of non-additive dominant effects in our study.

A second, less complex procedure for developing free-hybrids envisaged in Lodi implies the assessment of two-way or three-way free-hybrids derived from three narrowly-based germplasm pools issued from various cycles of phenotypic selection. These pools are: (i) one locally top-performing landrace from northern Italy, i.e. landrace 17 in Figure 1; (ii) one selection from the collection of Egyptian germplasm evaluated by Carelli *et al.* (2007) (featuring lower winter dormancy than the Italian landrace, and selected mainly for winter hardiness); (iii) one selection from semi-erect germplasm originated from Eastern Europe and from Spanish Mielga populations (featuring higher winter dormancy than the Italian landrace, and selected mainly for upright growth, relatively deep crown and purple flowers; Pecetti *et al.* 2006). The three-way hybrid would contemplate a 50% genetic background for the local landrace, which is expected to display greater adaptation to local conditions (Figure 5). The allegedly higher yield stability of the free-hybrid material (Brummer 1999) would also be assessed across contrasting growing conditions. The synthetic variety obtained by selection only within the local landrace would be the reference germplasm for testing the free-hybrids and verifying whether the possible increase of heterosis provided by hybridization with exotic material could offset the lower richness in locally-useful adaptive genes that the exotic material is expected to provide. This issue is of crucial importance when assessing the potential of free-hybrids, as their need for genetically-distant contributing germplasm requires the introduction of exotic germplasm and its selection for local adaptation. In our case, two cycles of phenotypic selection of the Egyptian germplasm pool were insufficient to reach an acceptable level of winter hardiness for this component of free-hybrids (Annicchiarico *et al.* 2009).

How to improve the forage quality?

Protein content and other quality aspects (e.g. digestibility) of lucerne forage are strictly related to the leaf/stem ratio at the harvest. Protein content is also affected by leaf age and health. Among-variety variation for protein content proved very
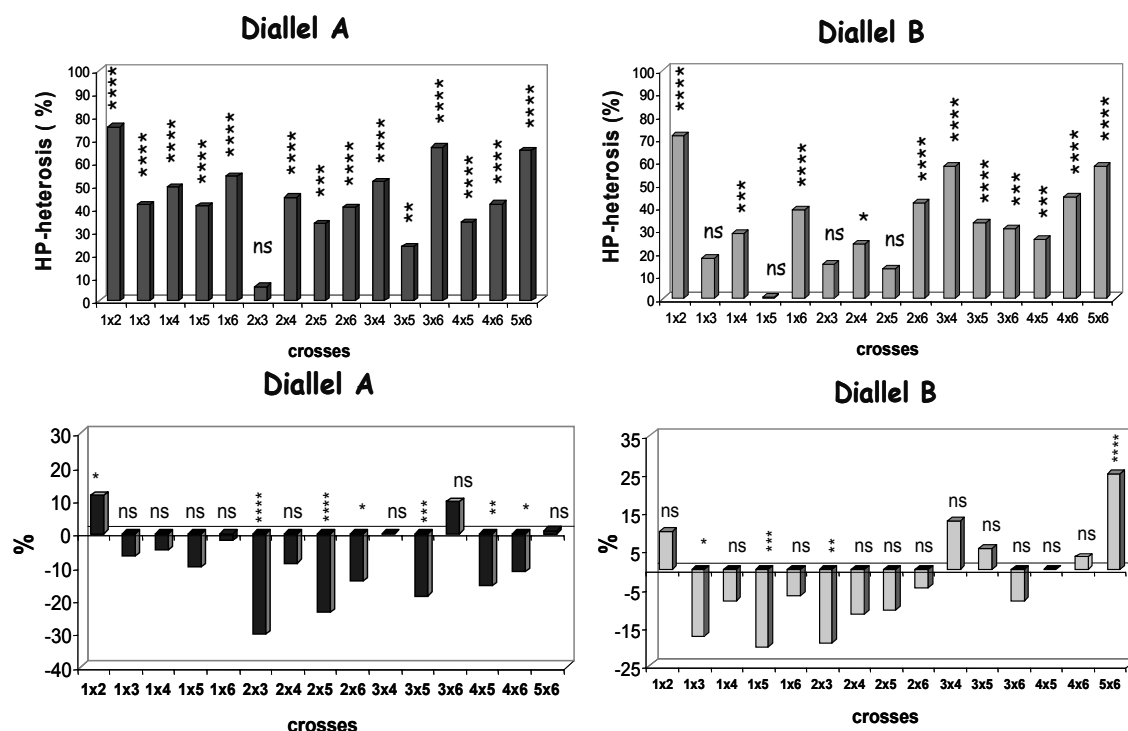
Figure 4. Dry matter yield over 10 cuts of double hybrids relative to: (i) the best parent (high-parent heterosis; upper diagrams), and (ii) the corresponding Syn4-generation synthetic (lower diagrams), for two independent diallelic sets (A and B)

low when sampling and analyses were made on leaves and stems of the same age, physiological stage and health (Rotili *et al.* 1991), while large variation occurred in relation to the stratigraphy of the lucerne stand and to the developmental stage (Rotili *et al.* 1992). However, investigations on the genetic structure of the leaf/stem ratio revealed that within-variety variation is much larger than among-variety variation for the leaf/stem ratio (Julier *et al.* 2000; Annicchiarico 2007c) as well as for forage yield-related traits (Julier *et al.* 2000; Annicchiarico 2006b), highlighting the paramount importance of selection within populations for exploiting lucerne genetic resources.

Quantitative genetic analyses performed on a large germplasm sample (125 genotypes used for comparison of selection schemes in Figure 2) confirmed the possibility for the simultaneous improvement of leaf/stem ratio and forage yield. These traits showed just a slight, non-significant inverse genetic correlations on the ground of parent-offspring regression ($r_g = -0.18$). Leaf/stem ratio showed sizeable genetic variation as well as high narrow-sense heritability, whereas forage yield displayed large genetic variation but fairly low narrow-sense heritability (Annicchiarico, unpublished results).

Alternative avenues for improving protein content and other quality traits could focus on specific morphophysiological traits. These include the tolerance to early (5% blooming) cutting (Rotili *et al.* 1991), the uncoupling of rate of growth and rate of development, the selection for higher leaflet number (Juan *et al.* 1993), and the modification of stem morphology towards a higher number of shorter internodes. Early mowing has a major impact on forage quality, but requires varieties which tolerate frequent cutting. Stem morphology is the result of the number of nodes and the length of internodes as well as the number and morphology of stem branches (Rotili *et al.* 1998), and variation for these traits is strongly influenced by environmental factors. However, interspecific hybridization between lucerne and other subspecies of the *M. sativa* complex can give rise to plant materials with modified stem morphology (Arcioni *et al.* 1994). A breeding program for stem morphology started in Lodi by crossing *M. sativa* genotypes of different origin with selfed progenies of plants derived from *M. sativa* × *M. falcata* somatic hybridization. We aimed at modifying the stem morphology towards an increased number of shorter internodes while maintaining the original

Table 5. Comparison of a short-internode synthetic variety vs. commercial cultivars for stem morphology, leaf/stem ratio and crude protein yield (values averaged across three harvests of the first cropping year)

Trait	Short-internode synthetic	Commercial cultivars
Stem height at the 1st reproductive node (cm)	72.7[a]	69.0[a]
No. of vegetative internodes	10.2[a]	8.2[b]
Internode length (cm)	6.9[b]	8.0[a]
Leaf/stem ratio	0.8[a]	0.7[b]
Protein yield/plant (g)	0.61[a]	0.54[b]

Row means with different letter differ at *P* < 0.05

total stem height and diameter, assessing the effect of the modified stem morphology on the leaf/stem ratio, protein content and protein yield. Two cycles of selfing were carried out in dense conditions on plants derived from the crosses. Positive selection was applied for plant dry matter yield, stem height and diameter, while applying divergent selection for the average internode length of the main stem. The S_2 individuals chosen with short and long internode length (SI and LI, respectively) were polycrossed by hand to obtain Syn1 and Syn2 generation synthetics. The comparison between Syn2 synthetics confirmed shorter internodes and higher number of internodes for SI relative to LI and some check varieties (Scotti *et al.* 2007). The modified stem morphology brought an increase in leaf/stem ratio and crude protein yield per plant (Table 5), supporting the interest in this selection approach. Inter-taxa hybridization within *Medicago sativa* complex could be an effective tool for enlarging the useful genetic variation in stem morphology.

Which opportunities for marker-assisted selection?

Various linkage maps of increasing density mainly based on SSR or AFLP markers have been developed for lucerne (e.g. Julier *et al.* 2003; Sledge *et al.* 2005) and then exploited for studying marker-trait associations, overcoming the difficulty represented by the tetrasomic inheritance. Mapping populations represented by F_1 or backcross generations have been used to detect Quantitative Trait Loci (QTL) for traits related to forage yield (e.g. Musial *et al.* 2006), and such investigations are expected to rise.

Just like top-performing germplasm and useful adaptive traits, also useful QTL and markers for forage yield are expected to be site-specific across a target region featuring large GE interaction for forage yield. Indeed, very poor consistency across two US sites was recently found for useful markers associated with higher lucerne forage yield (Robins *et al.* 2007) and persistence (Robins *et al.* 2008). These findings suggest that the assessment of opportunities for marker-assisted selection should be verified on a multi-environment basis, choosing contrasting test sites or test conditions on the ground of previous knowledge of GE interaction patterns across the target region.

An alternative method of QTL detection which has a special interest when breeding for an area characterized by one major stress (e.g. frost or drought) is based on the assessment of shifts in marker allele frequency from unselected to selected material through a bulk segregant analysis (Skinner *et al.* 2000; Castonguay *et al.* 2006). Its efficiency is expected to increase if the stress application can be controlled, the phenotypic selection of individual plants is performed through procedures which minimize the experimental error (e.g. by stratified mass selection), and several selection cycles can be performed (each followed by recombination). In comparison with the analysis of one F_1 biparental or backcross population, this method is more time-consuming and requires a denser genomic scan but has larger inference space, especially if the population undergoing selection has been formed or assembled so as to represent a wide genetic base. Another advantage of this approach is the concurrent selection of improved stress-tolerant germplasm.

Association genetics, also based on linkage disequilibrium between markers and phenotypic traits, has emerged as a tool to dissect the variation for complex trait down to the sequence level. The candidate-gene association mapping approach exploits Single Nucleotide Polymorphisms (SNPs) to infer genetic relationships with phenotypic vari-
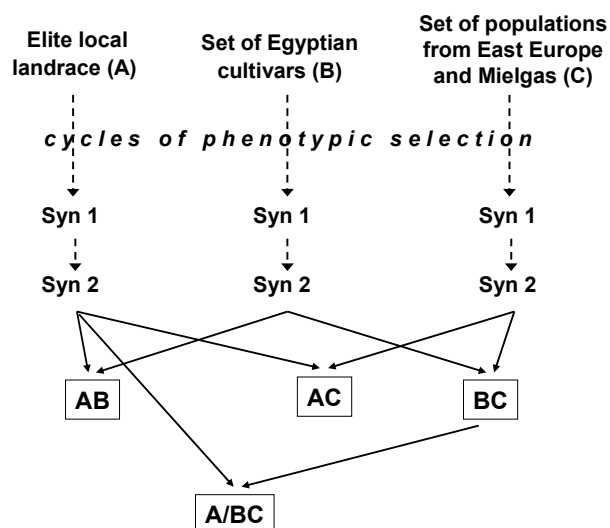
Figure 5. A simple procedure of two-way and three-way free-hybrid production in Lodi based on putative heterotic populations produced from S_0 parents

ation for specific traits, such as flowering date or plant length (Julier *et al.* 2009).

In the long run and along with opportunities for high-density marker evaluation, genomic selection (Heffner *et al.* 2009) may represent an alternative option also for this species (Brummer 2008). This approach, based on the statistical modelling of phenotypic values as a function of marker loci and subsequent stages of model validation, may be convenient when selection is performed on a well-defined genetic base.

How to exploit genomic information from *M. truncatula*?

Studies of functional genomics in *Medicago truncatula* have considerably boosted in recent years because of the importance of this model species. Collections of mutants which have become increasingly available are expected to help unravel the genetic control of important traits for many legume species. The *M. truncatula* consortium has made significant achievements in genome and Expressed Sequence Tag (EST) sequencing. This can fill the gap of information in the *M. sativa* genome sequence, as well as facilitate genetic and genomic studies in this species. For example, 64 out of 73 SSR molecular markers currently used in Lodi for lucerne diversity studies derive from *M. truncatula* EST and genomic libraries. Many SSR markers from *M. truncatula* are also mapped in *M. sativa* (Julier

et al. 2003), thereby facilitating molecular marker studies (identification of QTL, etc.).

Two mutant collections of *M. truncatula* have been established in Lodi in cooperation with CNR-IGV in Perugia (Porceddu *et al.* 2008) through insertional and chemical mutagenesis. These collections allow complementary approaches of forward and reverse genetics. We are currently focusing on pathways of secondary metabolites which are conserved in the *Medicago* genus, particularly the triterpenic saponin pathway, owing to the interest in saponin biological activities (natural biocide; probiotic; pharmacological activity). In particular, T_1 progenies of the collection obtained by insertional mutagenesis were phenotypically screened, identifying a mutant plant which lacks haemolytic saponins in the leaves. The mutation resulted in the loss of function of a novel member of the cytochrome P450 family. The collection obtained by chemical mutagenesis was screened for SNPs, recovering four alleles for the cytochrome P450 gene which were related to the absence of haemolytic saponin phenotype. Two of these mutants confirmed the absence of haemolytic saponins in the leaves, in agreement with the role of the reported gene in the pathway. This mutation may help clarify the biochemical pathway leading to the synthesis of saponins in *M. truncatula* leaves, thereby opening new opportunities for manipulating these compounds in the *Medicago* genus. In particular, we cloned a homologous P450 gene in *M. sativa*, and our ongoing purpose is verifying the role of this gene in the haemolytic saponin pathway of lucerne and using this gene in Eco-TILLING studies aimed at locating allelic series in lucerne natural populations. That would ultimately allow for defining specific molecular markers for marker-assisted selection of lucerne with modified saponin content and composition.

Information from *M. truncatula* on marker-trait association for major agronomic traits such as forage yield, persistence or tolerance to abiotic stresses (drought; low temperatures) is unlikely to be exploitable in lucerne, when considering the large differences between an annual and a perennial species for crucial adaptive traits (e.g. winter dormancy or root concentration of nitrogen reserves for regrowth and sugar reserves for frost tolerance; Volenec *et al.* 2002). In addition, metabolites produced by the same genes may have different functions in *M. truncatula* and *M. sativa* (Volenec *et al.* 2002), while differences due to the ploidy level may further contribute to the inconsistent genetic

control of some morphophysiological traits between the two species (Bingham *et al.* 1994).

The candidate-gene approach based on genomic information from *M. truncatula* may still prove useful to clone the orthologous genes in *M. sativa* and verify their variation in breeding material selected for contrasting agronomic or adaptive traits (Julier *et al.* 2009). However, there is a limit to the expected contribution of genomic tools developed for *M. truncatula* even for breeding of a relatively close species such as *M. sativa*. Concurrently, there is a serious risk that increasing funding of genomic research may occur at the cost of decreasing funding of essential research on other lucerne breeding issues, thereby jeopardizing lucerne breeding progress in the next few decades (Brummer 2004).

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