Ten years of research on Medicago at 'Instituto di Ricerche sul Miglioramento Genetico delle Piante Forraggere del CNR di Perugia'

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TEN YEARS OF RESEARCH ON MEDICAGO AT 'INSTITUTO DI RICERCHE SUL MIGLIORAMENTO GENETICO DELLE PIANTE FORAGGERE DEL CNR DI PERUGIA'

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ABSTRACT

In the last 10 years the Istituto di Ricerche sul Miglioramento Genetico delle Piante Foraggere (IRMGPF)'s research activity, has been moving gradually from traditional plant breeding to biotechnologies. Regarding traditional plant breeding of *Medicago sativa* the Institute has developed researches on 2n gametes in alfalfa and utilization of annual species of *Medicago* in genetic improvement of lucerne. Data on the presence of 2n gametes in diploid populations of *Medicago sativa* complex have been used either to increase the frequency of 2n gametes production, or to determine the mechanisms responsible for the formation of such gametes. The utilization of annual species of genus *Medicago* aimed to introduce in alfalfa traits for resistance to biotic and abiotic stresses.

The IRMGPF has begun different biotechnology researches. Two of these are the somaclonal variation in *Medicago sativa* in order to discover interesting phenotype for quantitative traits and *in vitro* selection to increase resistance to *Fusarium* wilt. The transfer of traits from the wild *Medicago* species has been carried out by somatic hybridization and embryo rescue. Embryo culture has been successful in producing hybrids between *M.sativa* and *M.rugosa*, while protoplasm fusion made obtainable hybrids *M.sativa* + *M.coerulea* and *M.sativa* + *M.arborea.* Somatic hybrid plants between *M. sativa* and *M. coerulea* have been RFLP finger printed to establish their nuclear composition. Furthermore the RFLP have been used to estimate the heterozygosity level of alfalfa populations and have been adopted for variety identification in *M.sativa.* Another research is the improvement of forage quality by genetic transformation. After preliminary experiments to set up the right working conditions for recovering at high frequency transgenic plants, goals are to produce lucerne genotypes bloat-safe and with an increase amount of sulphurous aminoacids.

Key words: *Medicago* sativa, unreduced gametes, *in vitro* selection, somaclonal variation, embryo culture, RFLP

INTRODUCTION

In the last 10 years the Istituto di Ricerche sul Miglioramento Genetico delle Piante Foraggere (IRMGPF)'s research activity has been moving gradually from traditional plant breeding to biotechnology application on the fodder crop species. Even if the former has given a large and crucial contribution to the production of cultivated varieties, the latter offers new tools for understanding the genetic complexity of forage species and for overcoming some traditional genetic barriers, so the combination of both approaches turns out to be a promising strategy to make further progress in the genetic improvement of these crop species.

As regards the genetic improvement of *Medicago sativa* the main objectives of the IRMGPF are 1) to take advantage of the existing variability in the cultivated varieties 2) to induce new variability and to transfer alien genes in this species and 3) to assess the genetic make up of plant genotypes.

For the first point the Institute has developed researches on 1a) 2n gametes; the research activity concerning the second point was 2a) Utilization of annual wild species of *Medicago* in genetic improvement of alfalfa 2b) Wide hybridization through protoplast fusion and embryo-rescue 2c)

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Somaclonal variation and 2d) Genetic transformation; for the third point researches have been carried out on the 3a) Use of molecular markers.

1A. 2n GAMETES IN ALFALFA

The possibility of 2n gametes utilization in the genetic improvement of alfalfa arises from the complexity of tetrasomic inheritance. In a cross-fertilizing autopolyploid such as alfalfa, heterozygosity can be maximized but not fixed by normal sexual means; as an alternative, the hybridization of 2n gametes produced by diploid hybrids can allow highly heterozygous and homogenous tetraploid varieties to be obtained (Dumbier and Bingham, 1975). Furthermore 2n gametes have been studied to explain the evolution of *M.sativa* complex and the germplasm transfer from diploid to tetraploid species of this complex.

Initial data on the presence of 2n gametes in diploid populations of *M.sativa* complex have been used to increase the frequency of 2n gamete production by cycles of phenotypic recurrent selection and to determine the cytological mechanisms responsible for the formation of such gametes (Mariani et al., 1992). A phenotypic recurrent selection program on the previously evaluated alfalfa diploid populations started in 1985. The results obtained showed that both male and female 2n gamete production were controlled by major and minor genes and that this trait was highly heritable. Therefore diploids with high frequencies of 2n gametes could be obtained by recurrent selection. The first two cycles of selection increased the percentage of plants producing 2n gametes and the frequency of 2n gametes per plant, while other two cycles of selection improved significantly these two parameters suggesting that the genes controlling this character were fixed in the considered diploid populations (Table 1). At the same time the plants selected indicated that the principle mechanisms responsible for 2n gametes formation could be grouped into two types: First Division Restitution (FDR) and Second Division Restitution (SDR). FDR-type is considered very important in the breeding of polysomic polyploids because with this mechanism it is possible to transfer a large amount of the heterozygosity of the diploid parent to the tetraploid offspring (Peloquin, 1983). It has been estimated that in *M. sativa* the value of the transmitted heterozygosity is about 80%. Five plants producing 4n pollen (Jumbo pollen) and 2n egg have been identified (Mariani *et al.*, 1993a). These mutants are quite interesting because, due to their functional male sterility, they can be used as 2n egg producers to obtain tetraploid progenies by bilateral sexual poliploidization (BSP). At the moment the research is in progress to produce BSP populations which will be evaluated for agronomic and physiological characters in comparison with natural tetraploids.

Table 1. Selection for 2n pollen and 2n egg production: effect of phenotypic recurrent selection on the average seed set per population and on the range of the seed set per plant

	a	b	c	d	e	f
2n egg						
Average seed set (%) [1]	4.26	11.46	26.4	7.14	37.73	42.40
Seed set per plant (%) (Range)	(0-96)	(0-75)	-0-160)	(0-20)	(1-191)	(0-196)
2n pollen						
Average seed set	0.38	4.97	13.11	3.32	33.54	51.54
Seed set per plant (%) (Range)	(0-50)	(0-45)	(0-105)	(0-10)	(1-115)	(8-115)

[1]Average seed set-(N. of seeds produced/N. of flowers pollinated) x 100
a: unselected population of 1st and 2nd cycles
d: unselected population of 3rd and 4th cycles
b, c, e, f: respectively are selected population of the 1st, 2nd, 3rd and 4th cycles

2A. UTILIZATION OF ANNUAL WILD *MEDICAGO* SPECIES IN THE GENETIC IMPROVEMENT OF ALFALFA

Annual species of genus *Medicago* can be used 1) to introduce traits for resistance to biotic and abiotic stresses in alfalfa and 2) as forage and cover crops in sustainable agriculture systems. Unfortunately many attempts to cross annual species with alfalfa have been unsuccessful, even if

more recently better results have been reached by using ovule and embryo culture (Mc Coy and Smith, 1986). For these reasons it's necessary to know the genetic structure of the annual species and their relationships with alfalfa. Furthermore, annual species are considered younger than the perennial ones and therefore they can be useful for studying the evolution of the cultivated species. At our Institute a cytogenetic study has been conducted on *M.murex* because it includes 2n = 2x = 14 and 2 n= 2x = 16 types. Cytological and morphological observations, together with complete intersterility, showed that these two types were quite different and could be considered as two distinct species (Mariani, 1990).

Later a study was started on other two annual species *M.rugosa* and *M.scutellata* to verify the origin of their unusual chromosome number, 2n = 4 x = 30, and to transfer their insect resistance to *M.sativa*. In fact, it seems that *M.rugosa* and *M.scutellata* have been derived by crosses between 2n = 14 and 2n = 16 species followed by doubling the chromosome number of the hybrids. Some controlled crosses among different annual diploid 2n = 16 and 2n = 14 species were carried out but, on the basis of morphological traits and on pollen structure , the plants obtained seemed to have been derived almost exclusively by selfing (Mariani and Tavoletti, 1993b). Based on these results research has been undertaken using cytogenetic and RFLP analysis to 1) characterize the annual diploid species previously indicated as the most closely related to the two species with 2n = 30 chromosomes (Classen *et al*, 1982) and 2) to establish the genetic distances between diploid species and *M.rugosa* and *M.scutellata*.

By the cytological and molecular analyses it was possible to identify four diploid species (*M.intertexta, M.muricoleptis*, *M.polymorpha* and *M.murex*) with the highest degree of genetic affinity with the two 2n = 30 species. The research is going on to verify the possibility of identifying the ancestors of those two species among the annual species more related to them.

2B. WIDE HYBRIDIZATION: PROTOPLAST FUSION AND EMBRYO-RESCUE

Intraspecific crosses have contributed largely to the genetic improvement of almost all the major crop species, but with the decreasing of the natural variability available, much attention has also been paid to gene exchange through species and genera. However wide crosses are often unsuccessful because of the mechanisms that interfere with the fertilization processes or in the survival of hybrid embryos. Somatic hybridization and embryo-rescue are valid tools for overcoming these obstacles. IRMGPF is carrying out a program of somatic hybridization to transfer into alfalfa important agronomical traits from wild species of genus *Medicago*, which are sexually incompatible with *M.sativa*. Viruses, bacteria and fungi are responsible for severe reduction in forage production and the wild *Medicago* species may be donors of disease resistance features. Moreover these species might be the source of useful agronomic traits such as adaptation potential and resistance to biotic and abiotic stresses (Arcioni, 1982).

During this program, it has not been so difficult to obtain hybrid calli through protoplast fusion, but in the early experiments these calli failed to regenerate plants (Damiani *et al.*, 1988; Pupilli *et al.*, 1991). According to our experience, in the genus *Medicago* regeneration from hybrid calli can be achieved either when both parental protoplasts are regenerable or when one fusion partner releases morphogenetic and highly dividing protoplasts and the other parent shows almost dormant protoplast which affect neither division nor regeneration of hybrid cells. In our Institute hybrid plants have been produced for the combinations *M.sativa* (2n=4x=32) + *M.coerulea* (2n=2x=16) and *M.sativa* + *M.arborea* (2n=4x=32), where *M.coerulea* is close to *M.sativa* while *M.arborea*, which is characterized by shrub habitus, drought resistance and winter growth, is considered the species most genetically distant from alfalfa. In alfalfa, the hexaploid level is more desirable than the tetraploid one, since the former assures a higher level of heterozygosity with a consequent slower decline in vigour due to self-fertilization (Lesins, 1975). Moreover somatic hybrids show a degree of heterozygosity higher than that of sexual hybrids, whose chromosome number has been doubled by colchicine treatment; in addition the somatic hybrids recombine also the cytoplasms of both parents.

To obtain hexaploid hybrids we electrofused callus protoplasts of *M.coerulea* which divided occasionally, with mesophyll protoplasts of *M.sativa* and the heterokaryons were visually recognized on the basis of their colour (red or green). In this way more than 50 plants were regenerated and their hybrid nature was confirmed by biochemical, molecular, cytological and morphological analysis

(Pupilli *et al.*, 1992). The hybrid and parental plants were grown in field and then evaluated for some morphological and agronomic traits (Table 2). For some characters such as blooming time, the hybrids did not differ from the *M.sativa* parent, while for growth habit they were intermediate and significantly different from both parents (Fig.1). The average number of stems per plant and the number of nodes on the main stem were much lower in the hybrids than in the parents. The effect of the reduced number of stems and nodes on the forage yield of hybrid plants was however counterbalanced by their higher leaf weight and stem diameter, so that leaf and stem weight per plant exhibited average values not too different from *M.sativa*, the more productive parent. However, the somatic hybrid plants are at least no worse than *M.sativa*. More recently we have produced somatic hybrid plants between alfalfa and *M.arborea*, but while the somatic hybrids involving members of the *M.sativa* complex showed chromosome stability and fertility, in this case the hybrids showed some perturbations at the molecular and cytological levels (lack of some parental bands, chromosome loss, etc.) and did not flower during the first two years of soil adaptation (Arcioni *et al.*, 1994). Preliminary analyses on somatic hybrids showed a large variability of some morphological traits such as size and shape of leaves while for the growth habit and the number of stoma per mm^2, the hybrids where intermediate to the parents. In addition in the somatic hybrids the internode length is shorter than in the parents and consequently the hybrids have more leaves than both parents (Nenz, in preparation) (Fig. 2).

Table 2. Field evaluation of agronomic traits of the somatic hybrid population

TRAIT.	MEAN SE	MS[1]	MC[2]
Main stem diameter	2.24 ± 0.039	2.10**	2.15*
Blooming time	16.98 ± 0.627	16.33ns	19.87**
Node number	12.12 ± 0.122	14**	13**
Leaflet length	2.35 ± 0.037	2.03**	1.70**
Stem length	56.04 ± 1.10	55.77ns	60.96**
Leaf weight/plant	34.41 ± 2.271	35.72ns	19.94**
Growth habit	3.36 ± 0.131	4.67**	1.00**

[1] MS = *M. sativa*
[2] MC = *M. coerulea*
* Values not significantly different for $P \leq 0.05$
** Values not significantly different for $P \leq 0.01$

Figure 1. Somatic hybrid phenotype: *M.sativa* (left), somatic hybrid (middle), *M.coerulea* (right).

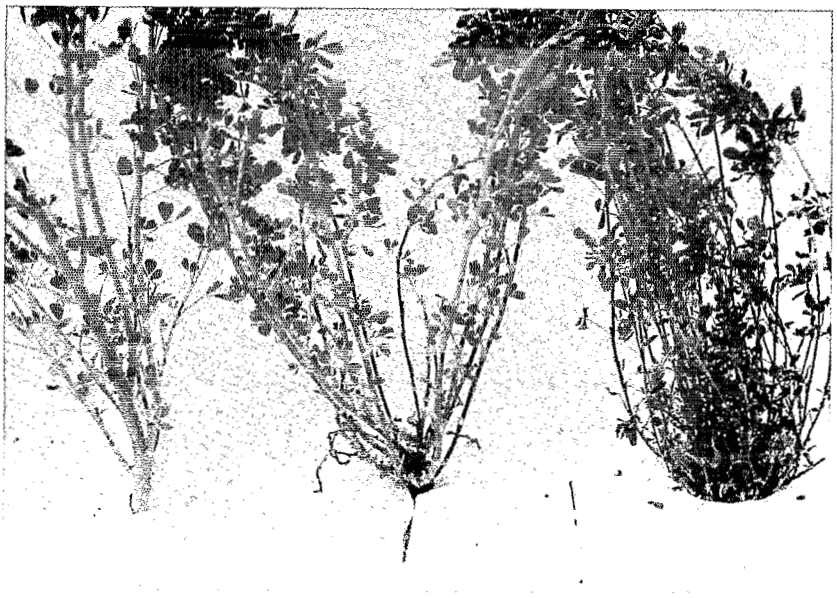

Figure 2. Growth habit of the somatic hybrid: *M.sativa* (left), somatic hybrid (middle), *M.arborea* (right).

Somatic hybrid plants *M.sativa* (2n=32) + *M.falcata* (2n=16) have been produced very recently and preliminary evaluations at molecular level have confirmed their hybrid nature , and in particular the southern analysis of the rDNA provides a lot of information on the hybrid genetic structure.

In general in the genus *Medicago* inter-specific somatic hybrids seem to be an interesting material for plant breeding since the parental genomes have almost been completely transmitted to the progeny, the frequency of meiotic alterations is low, the sexual progeny displays chromosome stability and the morphological traits are similar or superior to those of the tetraploid parents.

If the wide-crosses are prevented by post-zygotic mechanisms which interfere with the development of the hybrid embryo, the embryo-rescue technique can help the embryo to survive and to develop into a plant. In order to transfer into *M.sativa* the resistance to potato leaf hopper and alfalfa weevil typical of *M.rugosa* (2n = 30) interspecific hybrids have been produced through ovule-embryo culture due to the presence of glandular hairs (Piccirilli *et al.*, 1991). The hybrid nature of recovered plants was confirmed by biochemical and cytological analyses but they did not show the presence of glandular hairs.

2C. SOMACLONAL VARIATION AND *IN VITRO* SELECTION

Due to the capacity of producing new alterations in plants regenerated from cell cultures, somaclonal variation has been investigated to verify the possibility to obtain new variants for quantitative traits. The results showed that in *M.sativa* the *in vitro* culture didn't produce superior phenotypes respect to those present in the original population and the analyses of offspring revealed the hereditability of the modifications produced by *in vitro* treatment (Arcioni *et al.*, 1989). Similar observations have also been reported in *L.corniculatus* (Damiani *et al.*, 1990), and the failure in getting benefits from the somaclonal variation, is caused by the fact that the plant vigour is under the control of polygenic systems and is related to the level of heterozigosity (Bingham *et al.*, 1979), while the *in vitro* culture can produce modifications only in one or few genes.

On the other hand when cells carrying a particular alteration are selected, somaclonal variation can produce new genotypes of practical value. In fact in presence of culture filtrate of *Fusarium oxysporum* plants resistant to the fungus have been regenerated (Arcioni *et al.*, 1987) (Table 3). The *in vitro* approach can be effective for large-scale breeding programs aimed at recovering disease resistance plants, but the success in the selection greatly depends on the *in vitro* activity of the toxin

produced by the pathogens, because if the culture filtrate fails to affect cell growth it is not possible to perform any selection.

Table 3. *In vitro* responses to *Fusarium oxysporum* of some plants (R 1-4) regenerated from culture filtrate resistant calli, and susceptible (P_1, P_2) and resistant ($F_{208/05}$) controls

Plant designation	Average severity index	Significance of t-test (Selected Vs$F_{208/05}$)
R_1	0.7	n.s
R_2	1.0	n.s
R_3	0.9	n.s
R_4	2.5	**
P_1	2.5	**
P_2	2.5	**
$F_{208/05}$	1.0	

n.s: not significant
**: significant per $P \leq 0.01$

2D. MOLECULAR MARKERS

Molecular marker assisted selection of desirable genotypes, can remarkably shorten the length of breeding program (Tanskeyl *et al.*, 1989). Two classes of molecular markers are now well established for breeding purposes: RFLP (Restriction Fragment Length Polymorphism) and RAPD (Random Amplified Polymorphic DNA) both based upon polymorphism detectable at DNA level.

When the genetic information present in different plants is combined into new hybrids, it is essential to know their nuclear composition before their use in breeding programs. The somatic hybrid plants *M.sativa* + *M.coerulea* have been RFLP finger-printed to establish their genetic make up: although all of the chromosomes were present, an incomplete incorporation of the alleles of *M.coerulea* was observed (Pupilli *et al.*, 1994), and most probably this was due to chromosome rearrangements, occurred in *M.coerulea* genome during callus growth before fusion. In the polycross progeny of the somatic hybrids, the RFLP alleles contributed by both parents segregated in a Mendelian mode, suggesting a genetic stability of these plants.

In alfalfa the forage yield is positively correlated to both the heterozygosity level expressed as a percentage of tri and tetra allelic loci and the quality of genes and linkats (Demarly, 1963). The main obstacle in the genetic improvement for forage production in alfalfa is the difficulty in splitting them apart. In collaboration with "Istituto per le Colture Foraggere di Lodi" the RFLP have been used to estimate the heterozygosity levels of alfalfa populations subjected to recurrent cycles of selfing in a program aimed to select the most vigorous plants as a result of particular gene combinations rather than of a high heterozygosity level (Rotili, 1976). In this way relatively high yielding-homozygous plants can be isolated and crossed with each other, to produce an alfalfa variety that cumulates the two components (i.e. heterozygosity and quality of genes) of the forage yield.

The heterozigosity level is related to the number of bands detected on the autoradiographic films and no correlation has been observed between plant vigour and heterozigosity. About 20 plants have been selected in a S_2 generation for high vigour and low heterozigosity, and their polycross progeny will be used as starting material for the constitution of a synthetic high yielding alfalfa variety.

Reports on cultivar identification by molecular markers concerned mainly autogamous and vegetatively-propagated species, while little is reported on outcrossing seed-propagated species such as alfalfa. This is due to the high degree of intra-population variability of the latter species which makes it difficult to find cultivar-specific markers. Researches are in progress to assess the practical utilization of RFLP and RAPD in alfalfa to differentiate synthetic cultivars and local ecotypes adapted to the environment of Central Italy.

A study conducted on ten alfalfa populations indicated that, either varieties or ecotypes can be distinguished on the basis of statistically significant differences in allele frequencies. Furthermore, by bulking the DNAs of different individuals of the same cultivar, it is possible to compare different populations each represented by a unique sample. This last approach seems to be of practical use for its facility and reproducibility, and once many cultivar-specific markers are identified, they can be used for the certification of the alfalfa varieties (Table 4).

Table 4. Diagnostic RFLP hybridization fragments used in cultivar identification

P/E combination	Fragment N°	F1	Fz	R	As	N	M	E	Ra	Rg	A
		Fragment frequency (%)									
AluI/2B9	3	nd	100	100	100	100	100	100	100	57*	100
RsaI/1C5	10	100	94*	100	100	100	100	100	100	100	100
RsaI/3C3	5	20	7	23	27	53*	25	25	12	28	20
AluI/3C6	3	12	0	0	11	0	5	6	0	28*	0
AluI/3C6	4	0	7	6	5	7	5	0	100*	57	0
AluI/3C6	8	0	0	25	100*	7	10	13	57	0	20
AluI/3C6	9	100	100	100	100	100	100	100	100	100	93*
TaqI/1H11	2	18	76*	19	11	33	10	15	14	0	nd
TaqI/1H11	3	100	100	100	100	100	90	100	71*	100	nd
TaqI/1H11	4	12	15	9	0	10	10	5	57*	14	nd
TaqI/3F3	9	0	0	0	0	0	10*	0	0	0	0

F1 = Florida, Fz = Furez, R = Romagnola, As = Ascolana, N = Natsuwakana, M = Maremmana, E = Europe, Ra = Rambler, Rg = Rangelander, A = Adriana
Values significantly different for P = 0.05 (*)

3A. GENETIC TRANSFORMATION

Genetic transformation has been considered an important aspect of alfalfa molecular biology since the early 80's. With this biotechnology tool, it is possible to transfer alien genes into the plant genome in order to obtain transgenic plants.

The genetic transformation of *M.sativa* and *M.arborea* has been performed with *A.rhizogenes* and *A.tumefaciens* (Spanò *et al.*, 1987; Damiani and Arcioni, 1991; Pezzotti *et al.*, 1991) and transgenic plants resistant to the antibiotics kanamycin and hygromycin have been obtained. These plants can be utilized as parents in experiment of somatic hybridization so that the resulting hybrid calli could be selected in media supplemented with the antibiotics. It has been observed that the recovery of transgenic plants is higher following transformation with *A.rhizogenes* than *A.tumefaciens*, but the Ri genes of the T-DNA (the *A.rhizogenes* DNA normally inserted in the genome of the host plant) causes alterations on the phenotype of the transgenic plants. Ri transgenic alfalfa plants showed abnormalities for different traits: number of stems and leaves, internode length, morphology of the root apparatus (Fig. 3a,b). Furthermore the transformed plants have lost their perenniality, in fact in field conditions they died after flowering.

At the moment genetic transformation has been utilized to improve forage quality in alfalfa. A first approach consists in increasing the contents of proteins rich in sulfur aminoacids and for this purpose a maize gene coding for a seed storage protein rich in cysteine and methionine has been transferred in *M.sativa* and, as model systems, in tobacco and *L.corniculatus.* To improve the expression of the transgenes in the new genomes, we are testing some constructs carrying different coding and promoter regions. Initial results showed a good transcription of the introgressed gene, while some difficulties are present at the translation level.

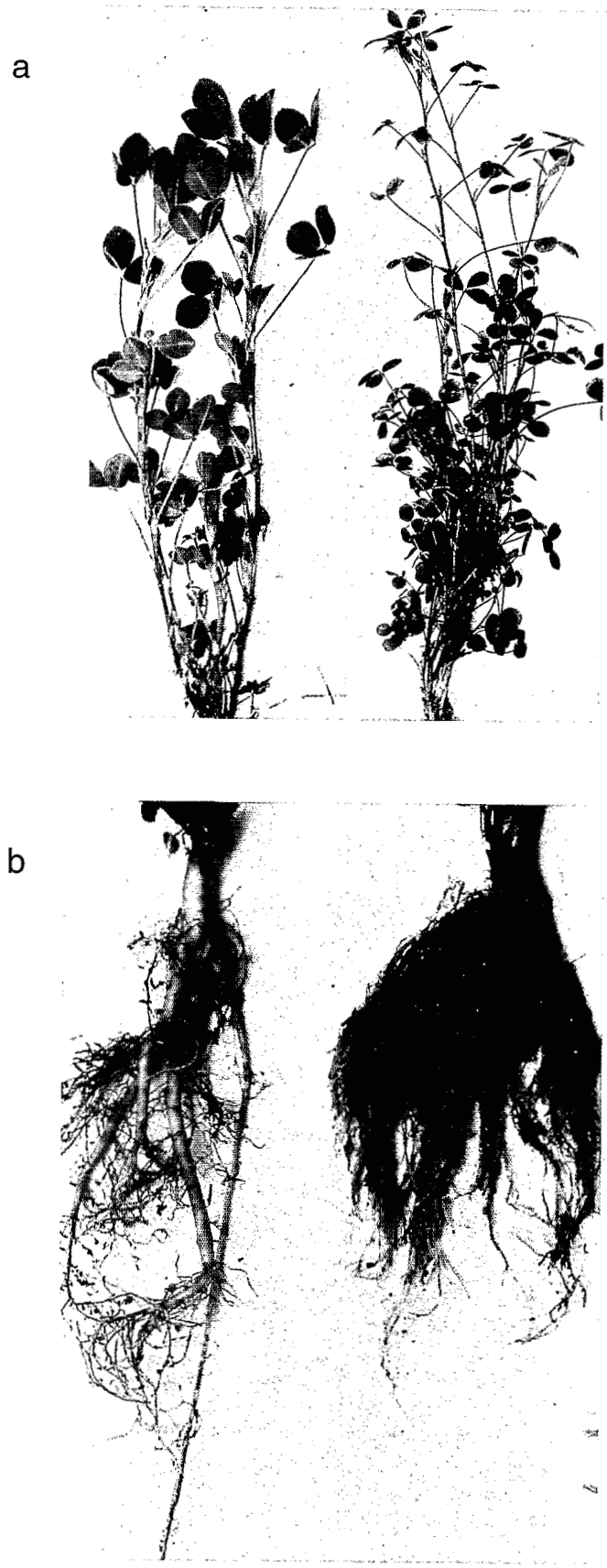

Figure 3. Stems and roots of the same genotype of *M.sativa* not transformed (left) and transformed (right) with *A.rhizogenes*.

A further application of genetic transformation concerns the improvement of forage quality through the introduction into alfalfa of the DNA sequences responsible for the synthesis of leaf condensed tannins (CT). As a matter of fact high contents of CT prevent the spontaneous intake by the animal, but the total lack of these compounds in forage legumes causes problems of bloating in ruminants and reduces the nutritive value of forage. The introduction into *L.angustissimus* and *L.corniculatus*, two species with leaf CT, of the "Sn" maize gene that regulates the synthesis of anthocyanidins (anthocyanidins and tannins have a common biosynthetic pathway) produces an increase in the roots and a decrease in the leaves of CT (Damiani *et al.*, in preparation).

These findings, other than offering a new strategy to improve those species where the excess of tannins is a limiting factor for forage appetition, could allow us, by comparing RNA of transformants and control plants, to identify those genes, still unknown, but responsible for tannin synthesis to be later introduced in tannin negative species such as alfalfa.

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