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Somatic hybrid plants between the forage legumes *Medicago sativa* L. and *Medicago arborea* L.

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Abstract Interspecific somatic hybrid plants were obtained by symmetrical electrofusion of mesophyll protoplasts of *Medicago sativa* with callus protoplasts of *Medicago arborea*. Somatic hybrid calli were picked manually from semi-solid culture medium after they were identified by their dual color in fluorescent light. Twelve putative hybrid calli were selected and one of them regenerated plants. The morphogenesis of the somatic hybrid calli was induced by the synthetic growth regulator 1,2 benzisoxazole-3-acetic acid. Somatic hybrid plants showed intensive genome rearrangements, as evidenced by isozyme and RFLP analysis. The morphology of somatic hybrid plants was in general intermediate between the parents. The production of hybrids by protoplast fusion between sexually incompatible *Medicago* species is related to the in vitro responsiveness of the parental protoplasts. The possibility of using somatic hybrid plants in alfalfa breeding is discussed.

Key words *M. sativa* · *M. arborea* · Protoplast electrofusion · Somatic hybrids · Genome rearrangements

Introduction

Medicago sativa (2n=4x=32; alfalfa, lucerne) is the world's most important forage crop (Michaud et al. 1988) and, as a perennial growing in different environments, its forage production is affected by biotic and abiotic stresses. Between annuals and perennials (Quiros and Bauchan 1988) the genus *Medicago* comprises more than 60 different species, which contain most of the genetic resources necessary to overcome the stresses faced by cultivated alfalfa (Isaac 1957; McEvans 1966; Mariani et al. 1978). However, the possibility of transferring useful traits into *M. sativa* by sexual crosses is restricted to strains with the same ploidy level (Mariani 1968) and belonging to the *M. sativa* complex; consequently, the utilization of wild *Medicago* species as germ-plasm sources for the genetic improvement of alfalfa requires the overcoming of sexual barriers through somatic hybridization. This method could also be used for exploiting the production of tetraploid plants by somatic fusion of two unrelated, highly heterozygous and genetically improved diploid plants (Dumbier and Bingham 1975). In any cultivar of *M. sativa* it is possible to find genotypes able to regenerate from different plant organs (Arcioni et al. 1990) and a number of cases of protoplast fusion with other *Medicago* species have been reported (Arcioni et al. 1994). But hybrid plants able to reach maturity have only been obtained with the combinations *M. sativa*+*M. falcata* (2n=4x=32) (Téoulé 1983; Mendis et al. 1991) and *M. sativa*+*M. coerulea* (2n=2x=16) (Pupilli et al. 1992); in other words, between species of the same *Medicago sativa-falcata-coerulea* complex (Quiros and Bauchan 1988) which, though having different ploidy levels, can be made sexually compatible by the use of unreduced gametes (Bingham 1979). The present paper reports somatic hybridization between *M. sativa* and *M. arborea* (2n=4x=32), two sexually incompatible and phylogenetically distant species. *M. arborea* is the oldest species of the genus *Medicago* and the only one with a woody growth habit. It is unlikely to have been involved in the origin of the present herbaceous Medicagos (Lesins and Lesins 1979). The main features of agronomic interest in *M. arborea* are drought resistance and the absence of summer and winter dormancy. It is used for pasture (Corleto et al. 1980) in dry lands, like the Mediterranean countries, where it can ensure an appreciable forage production in winter and in summer when the perennial *Medicago* species are dormant and the annual ones have already completed their biological cycle. In addition, *M. arborea* is resistant to bacterial wilt (*Corynebacterium insidiosum*; Renfro and Sprangue 1959) and to both races of anthrachnose (Elgin and Ostazeky 1982). Up to now the useful traits present in the wild *Medicago* species, and suitable for the

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genetic improvement of alfalfa, have not been cloned. The most promising method for transferring appropriate traits into *M. sativa* is by somatic hybridization through protoplast fusion.

Materials and methods

Plant material

Genotype R15 of *M. sativa* cv "Rangelander" (2n=4x=32), selected for high regeneration capability through somatic embryogenesis, was obtained by courtesy of Dr. Larkin (CSIRO, Plant Industry, Canberra). It was propagated by cuttings and used as a source of mesophyll protoplasts. Seeds of *M. arborea* (2n=4x=32), harvested from a mesophyll protoplast-derived plant (Mariotti et al. 1984), were surface sterilized (20 min with 0.1% w/v sodium lauryl sulphate, 20 min with 1.2% v/v sodium hypochlorite, three rinses with sterile distilled water) and germinated on agar-solidified (0.8%, w/v) growth-regulator-free MS medium (GRFMS, Murashige and Skoog 1962) at 1/4 strength under fluorescent light at 27 $\mu E\ sec^{-1}\ m^{-2}$, 22°C ±1, 21 h photoperiod (culture conditions A).

Callus induction

Hypocotyl sections (0.5 cm long), excised from 1-week-old sterile seedlings of *M. arborea*, were plated on UM (Uchimiya and Murashige 1974) agar-solidified medium with 3 mgl^{-1} of 2,4-D and 0.25 mgl^{-1} of kinetin and incubated under culture conditions A, subculturing every week.

Protoplast isolation

Mesophyll protoplasts of *M. sativa* were isolated from 5–7 day-old leaflets of plants grown in the cabinet at 216 $\mu E\ sec^{-1}\ m^{-2}$, 20°C, 12 h photoperiod, 80% relative humidity (culture conditions B) following the procedure described by Pupilli et al. (1991) except that the enzyme mixture consisted of 20 gl^{-1} of Cellulase Onozuka R10, 5 gl^{-1} of Macerozyme, 1 gl^{-1} of Pectolyase Y-23 in CPW salt solution (Frearson et al. 1973) with 100 gl^{-1} of mannitol, pH 5.8. Hypocotyl-derived calli of *M. arborea* were dispersed in UM liquid medium (5 g of callus in 50 ml of liquid medium, 2 days on a rotary shaker at 150 rpm; culture conditions A), then the cells were collected by centrifugation (300 g, 6 min) and re-suspended in the enzyme mixture (1 ml of packed cells in 5 ml of enzyme solution) consisting of 5 gl^{-1} of Cellulase Onozuka YC, 20 gl^{-1} of Driselase, 12 gl^{-1} of Pectinase, 3 gl^{-1} of Pectolyase Y23 in CPW salt solution containing 100 gl^{-1} of mannitol and 5 mgl^{-1} of fluorescein isothiocyanate (FITC), pH 5.8. Cell-wall digestion was performed on a rotary shaker (40 rpm, 5 h, 28°C, dark) and protoplasts were isolated as reported by Pupilli et al. (1991). Both mesophyll and callus protoplasts were mixed in the ratio 1:1 and re-suspended in the electrofusion solution (0.6 M mannitol, 0.2 mM $CaCl_2 \cdot 2H_2O$, 0.6 mM histidine) at a density of 3×10^5 protoplasts ml^{-1}.

Protoplast electrofusion and culture

The electrofusion procedure (with the exception of a field strength of 180 Vcm^{-1} and a pulse voltage of 2 100 Vcm^{-1}), the culture of fusion products, the selection of heterokaryons and their culture until the callus stage were all performed as reported by Pupilli et al. (1992).

Plant regeneration

The putative hybrid calli were grown for 2 months on UM agar-solidified (0.8 w/v) medium with 2 mgl^{-1} of 2,4-D and then transferred onto CDM1-BOA regeneration medium consisting of MS with 1 mgl^{-1} of 2i-P, 0.5 mgl^{-1} of 1,2 benzisoxazole-3-acetic acid (BOA) and 20 gl^{-1} of sucrose. As soon as embryo-like structures appeared on the callus surface these were transferred with a small portion of surrounding callus onto different media for secondary embryo formation: CDM (MS with 1 mgl^{-1} of BA and 0.1 mgl^{-1} of NAA), GRFMS, and CDM1 (MS with 1 mgl^{-1} of 2i-P and 0.1 mgl^{-1} of IAA). At each sub-culture, performed every 10 days, the embryo-like structures were separated from the callus mass and cultured separately with a small piece of callus; well-developed shoots appeared after 4–5 sub-cultures and were transferred into Magenta vessels (6×6×10 cm) containing 50 ml of agar-solidified root-induction medium. Different rooting media were tested: GRFMS, RL (Phillips and Collins 1979) without growth regulators, MS1 (MS with 1 mgl^{-1} of GA_3) 1/4 strength, MS2 (MS with 3 mgl^{-1} of GA_3) 1/4 strength, RL1 (RL with 1 mgl^{-1} of GA_3) and RL2 (RL with 3 mgl^{-1} of GA_3). Rooted plantlets were transferred into soil when 5–10 cm tall, maintained in a growth cabinet under conditions B and covered with a plastic bag to prevent desiccation. After 3 weeks of acclimatization the plants were transferred to the greenhouse.

Hybrid recognition and characterization

Esterase isozyme analysis of the putative somatic hybrids and regenerants from protoplasts of parental plants were performed according to Pupilli et al. (1992).

The chromosome number of seven plants regenerated from a putative hybrid callus was determined in root tips of cuttings, following the procedure of Piccirilli et al. (1988).

RFLP analysis was carried out according to Pupilli et al. (1995) on five hybrids and on ten plants regenerated from protoplasts of each parental genotype. Eight probes were used in combination with three restriction endonucleases (*Alu*I, *Rsa*I and *Taq*I) to screen polymorphism between one hybrid and two parental plants, taken as representative of each population mentioned above, while a subset of five probe/enzyme combinations was utilized for detecting intra-genotypic variation.

Ten cuttings of the genotype R15 of *M. sativa* and ten seed-derived plants of *M. arborea*, together with 14 hybrid plants regenerated from the same fusion event, were maintained in the greenhouse and evaluated for the following morphological traits: (1) number of stems per plant, (2) diameter (mm), (3) number of internodes and (4) length (cm) of the longest stem; plant vigour was evaluated by: (5) leaf mass, determined by scoring from 1 to 5; (6) total green matter (stem + leaves) by scoring from 1 (weak plant) to 9 (the most vigorous plant); (7) number of leaves of the upper part (15 cm) of the longest stem; (8) number and (9) weight of the trifoliate leaves on the central node of the longest stem. The central leaflet of the largest trifoliate leaf, in the position mentioned above, was also scored for (10) number of teeth, (11) tooth depth (mm), stomata length (µm) (12), width (µm) (13) and number per mm^2 (14) and number of hairs per mm^2 (15). Data were submitted to ANOVA using the GLM procedure of the SAS (1988) program.

Results and discussion

Protoplast culture and plant regeneration

M. sativa mesophyll protoplasts were released with a yield of about 5×10^6 protoplasts g^{-1} of leaf material. They were characterized by a marked tendency to undergo mitotic divisions (more than 70% of the plated protoplasts started to divide after 10 days of culture) and form mini-colonies (40% of plating efficiency) that resembled embryo-like structures at early stages of development. When these embryo-like structures were picked and transferred onto KM8 (Kao and Michayluk 1975) solid medium and incubated

under culture conditions A, almost all de-differentiated into callus that subsequently produced well-developed plantlets when cultured in succession on KM8, UM1 and CDM1 solid media. More than 90% of protoplast-derived mini-calli were able to regenerate plants through indirect embryogenesis while only few primary embryos developed into plants. *M. arborea* callus protoplasts were released with a yield of about 8×10^4 protoplasts ml^{-1} of packed cell volume. Protoplast release was strongly affected by callus age and the duration of liquid culture: the best protoplast source was the friable pale-yellow callus developed after 6–8 weeks of culture and consisting of actively growing clusters of 10–30 cells. Longer intervals of sub-culture produced calli with brownish parts that released only dead cells. Similarly, periods of liquid culture longer than 3 days increased the percentage of large and brownish cells unable to release viable protoplasts. Callus protoplasts of *M. arborea* were mostly derived from clusters of small cells and started to divide 3–4 days after plating with a frequency of 60–70%. After 1 month of culture in semi-solid medium, about 80% of the protoplasts that had undergone the first mitotic division developed into mini-colonies of 30–50 cells. These mini-colonies were picked manually and cultured in the medium sequence KM8, UM1 and CDM1. After 1–2 months in CDM1 the first shoots appeared in 30% of the plated calli. Shoots easily rooted on GRFMS medium.

The yield of heterokaryons ranged between 6–9% of the total plated protoplasts, and their capability to undergo mitotic division was strongly affected by the plating density and viability of nurse parental protoplasts. If the final plating density on the semi-solid nurse culture medium was lower than 1×10^5 protoplasts ml^{-1}, the division frequency of the parental protoplasts dropped from 70 to 10% and from 60 to 5% for *M. sativa* and *M. arborea* protoplasts respectively, and none of the 200 heterokaryons cultured at such a density survived beyond 10 days after fusion. Conversely, at a plating density of $1.5–2\times10^5$ protoplasts ml^{-1} almost 10% of the heterokaryons scored started to divide 4–5 days after fusion, and most of them underwent the second and third mitotic divisions. After 1 month of culture, 5 out of 150 heterokaryons considered had grown enough to be picked; three survived the transfer but only one regenerated plants. Embryo-like structures appeared after 1 month of culture of the putative hybrid callus in CDM1-BOA medium. When these structures were transferred to CDM medium, an abundant callus proliferation was noticeable and the differentiated structures reverted into callus. On GRFMS the embryo-like structures assumed a brownish color and died within 2 months, and only CDM1 proved effective in inducing the formation of secondary shoots capable of further development. The somatic hybrid plants *M. sativa+M. arborea* regenerated after 1 year of culture and morphogenesis occurred only after adding synthetic growth regulator BOA to the regeneration medium. The effect of BOA on morphogenesis was similar to that of IAA but the former is reported to be more active (Branca et al. 1993). Such a long period of culture is probably responsible for the reduced morphogenetic capacity of the hybrid callus compared to the parents. When the shoots induced in CDM1 showed fully expanded trifoliate leaves, they easily rooted in RL medium after 6 weeks of culture. MS1 and MS2 media induced the formation of a few roots with a slow elongation rate, while RL1 and RL2 promoted the appearance of new leaflets but did not stimulate rooting.

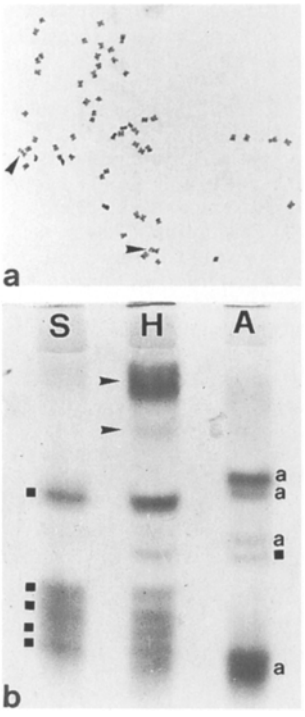

Fig. 1a, b Cytological and biochemical characterization of *M. sativa+M. arborea* somatic hybrids. Panel **a**: metaphase chromosomes of the somatic hybrid; *arrowheads* indicate satellite chromosomes. Panel **b**: esterase pattern of *M. sativa* (*S*), *M. arborea* (*A*) and their somatic hybrid (*H*); *arrowheads* indicate bands present in the somatic hybrid pattern only, *squares* indicate parent-specific bands retained in the somatic hybrids and the *letter a* indicates bands of *M. arborea* missing in the somatic hybrids

Characterization of hybrids

The hybrid plants showed a large variability for all the traits considered while no variation was evident among plants regenerated from protoplasts of each parent. A moderate loss of chromosomes was observed: six hybrids had 56 chromosomes, eight less than the sum of the two genomes, and one hybrid had 57. However, the small size and morphological similarity of the parental chromosomes make it impossible to determine the origin of the missing chromosomes (Fig. 1a). In the esterase electrophoretic banding pattern a selection was observed against the bands of *M. arborea*: whereas all the *M. sativa*-specific bands were clearly recognizable in the hybrid pattern (bands indicated by squares, lane S) only one band specific to *M. arborea* was retained (band indicated by a square, lane A). The absence of some parent-specific bands in the hybrid pattern (the bands indicated by a, lane A) coupled with the presence of bands which were absent in the parents (bands indicated by the arrows, lane H) excluded a chimaeric origin for the regenerated plants.

A high level of polymorphism was detected for RFLPs between the parental plants and, since no variability was

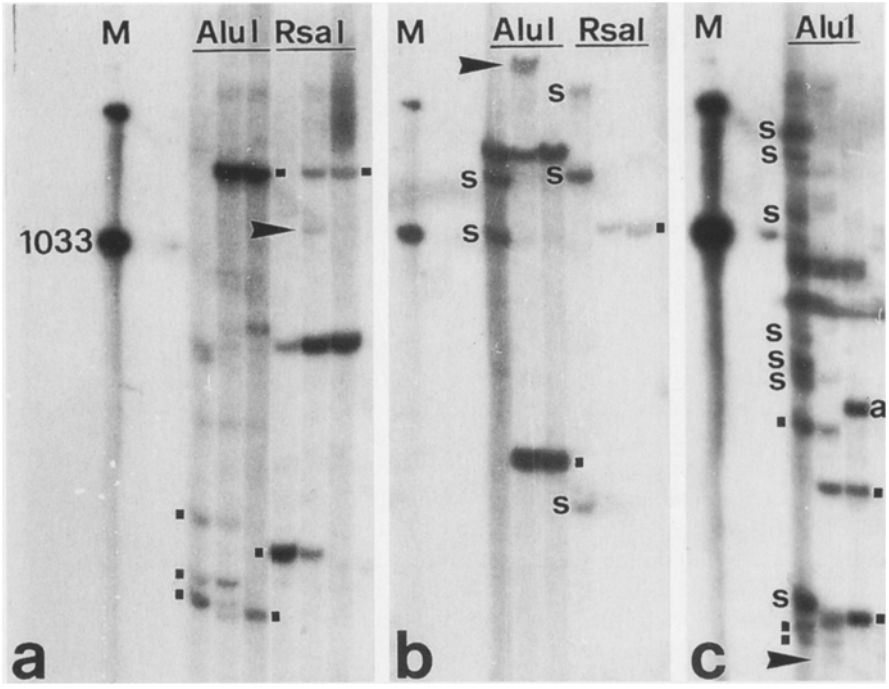

Fig. 2a–c RFLP analysis of somatic hybrids and parental plants. Panels **a**, **b** and **c** represent the hybridizing banding patterns of the genomic probes 3F3, 1H11 and 2G4 respectively; for each restriction enzyme, indicated on the top, lanes corresponding to the digested DNA of *M. sativa*, the somatic hybrid, and *M. arborea* are represented from left to right. *Arrowheads* indicate recombinant fragments; *s* and *a* respectively indicate *M. sativa*- and *M. arborea*-specific fragments missing in the somatic hybrids; and *squares* indicate parent-specific fragments retained in the somatic hybrid pattern. Molecular-weight markers (*M*) are expressed in base pairs

Table 1 RFLP analysis of parental and somatic hybrid lines

Lines	Number of					
	Probes	Probe/enzyme combinations	Total bands	Parent-specific bands	Missing bands in the somatic hybrids	Hybrid-specific bands
M. arborea	8	21	77	46	26	
M. sativa	8	21	98	67	22	
Hybrid	8	21	103	–	–	7

observed among plants regenerated from each parental and hybrid line (data not shown), two protoplast-derived parental plants and one somatic hybrid were used for RFLP analysis. From a survey of eight probes with three restriction enzymes (21 probe/enzyme combinations), it appeared that 38% of the total parental bands (175) were specific to *M. sativa* and 26% to *M. arborea* (Table 1). The presence of bands specific to both parents in the hybrid pattern confirms beyond all doubt the hybrid nature of the regenerated plants. Approximately one-third of the *M. sativa*-specific bands and half those of *M. arborea* were absent from the pattern of the somatic hybrids and seven bands specific to the somatic hybrid plants were scored (Table 1). Examples of all the above mentioned situations are given in Fig. 2. The large genome rearrangements observed in the hybrid plants could be related either to the duration of in vitro culture or to molecular and/or physiological mechanisms associated with the combination of parental genomes. However, the lack of variation for RFLP among five different somatic hybrid plants indicated that genome rearrangement took place before regeneration. Further hybridization experiments need to be performed to assess the effect of in vitro culture on genome rearrangements of hybrid plants. Chromosome loss could account for the absence of parental esterases and RFLP bands, but the lack of parental bands in the hybrid plants is not necessarily correlated with chromosome loss (Pupilli et al. 1995). The appearance of new bands in the hybrid pattern indicated that genome rearrangements were not only due to chromosome loss, but also to intensive mutational events, such as gene conversion, unequal crossing-over, gene duplication etc. which could be responsible for any gain, or loss, of restriction sites and, consequently, for the lack of parental bands and the appearance of new ones.

Morphological analysis

M. sativa and *M. arborea* differ very distinctly in many morphological traits. *M. arborea* is a shrub with an erect habit ensured by a lignified single stem; it is an evergreen species actively growing and flowering in winter. In contrast, *M. sativa* is a perennial herbaceous species characterized by several upright stems originating from the

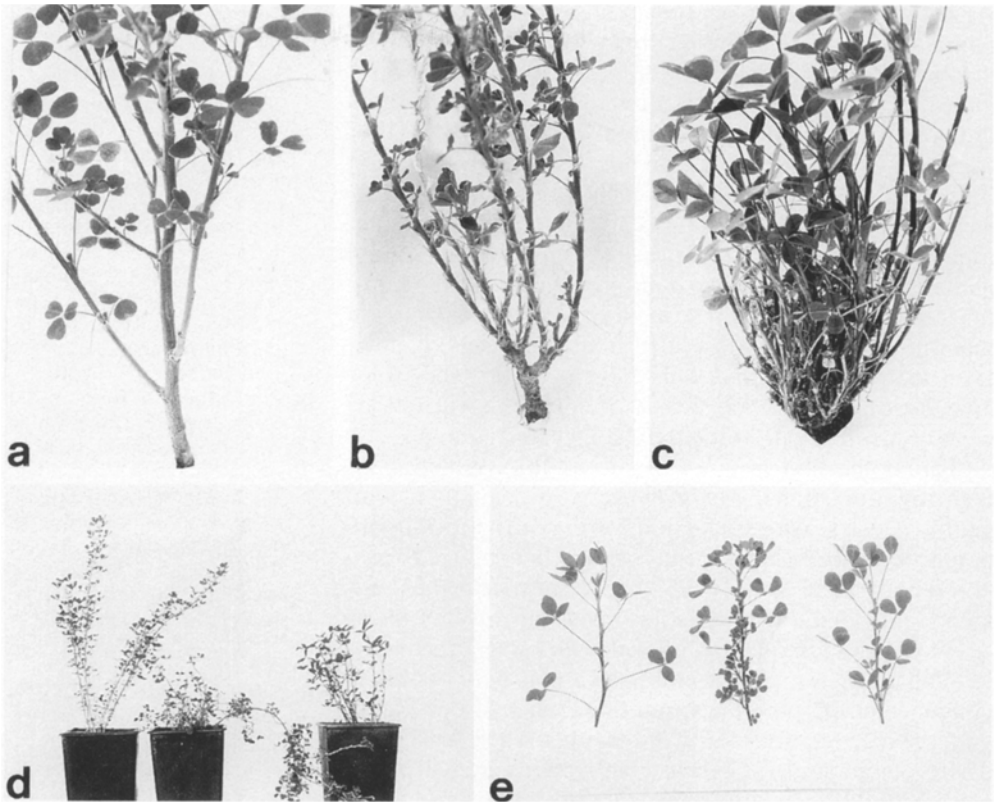

Fig. 3a–e Plant morphology of somatic hybrids and their parents. Panels **a–c**: stem proliferation at the crown of *M. arborea* (**a**), somatic hybrids (**b**) and *M. sativa* (**c**). Panel **d**: plant habit of *M. arborea* (left), somatic hybrids (middle) and *M. sativa* (right). Panel **e**: stem morphology of *M. sativa* (left), somatic hybrids (middle) and *M. arborea* (right)

crown; it is actively growing in spring and autumn, dormant in winter, and has a limited growth in summer. At maturity, *M. arborea* is 1.5–2 m high while the longest stems of *M. sativa* rarely exceed 1 m. For each of the above mentioned traits the hybrid plants were intermediate between the parents and showed: 6–10 stems per plant, originating from a crown-like structure (Fig. 3a-c); an active growth in winter, like *M. arborea*, and near-dormancy in summer, like *M. sativa*; some stems as long as those of *M. sativa* and a few (1 to 3 per plant) with an intermediate length (Fig. 3d).

All these traits made the hybrids easily distinguishable from the parental plants. Similar observations were made for leaf shape which appeared intermediate between the heart-shaped leaves of *M. arborea* and the oval-shaped ones (length twice the width) of *M. sativa*. The internode number in somatic hybrids was not significantly different from that of *M. sativa*, and in the upper part of the stems the nodes were very close to each other, conferring a characteristic leafiness to the plants (Table 2, Fig. 3e). As a result, the longest stems became prostrate, probably because their upper part was too heavy for stems that were not sufficiently lignified (Fig. 3d). The somatic hybrids had a rather vigorous aspect and their lower number of stems per plant compared to *M. sativa* (Table 2) was in part counterbalanced by their slightly higher leafiness, so that the apparent total green matter of the somatic hybrids did not differ significantly from that of *M. sativa*. The number of leaves on the main stem was significantly higher in the somatic hybrids (Table 2), while the number and weight of leaflets at the median internode of the main stem showed similar values in the parents and hybrid plants. The leaf margins of *M. arborea* were in general smooth, *M. sativa* showed toothed leaves and the somatic hybrids had more

Table 2 Morpho-agronomical traits evaluated in *M. sativa* (Ms), somatic hybrids (Sh) and *M. arborea* (Ma). Values followed by the same letter did not differ for $P \leq 0.05$

Trait	Ms	Sh	Ma
Number of stems	19a	6b	2b
Leaf mass (score 1 to 5)	1.67a	4.19a	3a
Total green matter (score 1 to 9)	5b	4.24b	9a
Stem diameter (mm)[a]	2.07b	2.11b	5.1a
Internode number[a]	13.67b	16b	28.33a
Stem length (cm)[a]	33b	25.91b	43.50a
Leaf number (upper 15 cm)[a]	17b	30.91ab	17b
Leaf number (median node)[b]	4.67a	5.95a	10.67a
Leaf weight (g)[b]	0.60a	0.79a	0.96a
Tooth number/leaflet[c]	13b	19.54a	5.33c
Tooth depth (mm)[c]	0.34a	0.25b	0.05c
Hair number/mm^2[c]	22b	16.87c	39.56a
Stomata number/mm^2[c]	94.67a	35.14b	23.11c
Stomata length (μm)[c]	24.42a	31.35a	23.10a
Stomata width (μm)[c]	15.84b	15.84b	19.47a

[a] Traits recorded on the longest stem
[b] Traits refer to the number and weight of trifoliate leaves of the median internode of the longest stem
[c] Traits recorded on the median leaflet of the largest trifoliate leaf on the position mentioned above

teeth, though less deep ones, than *M. sativa* (Table 2). The number of hairs was significantly lower in somatic hybrids than in the parents, while the number of stomata was intermediate. Stomata length and width in the somatic hybrids did not differ significantly from those of the parents (Table 2). The somatic hybrids did not flower during the first 2 years of soil acclimatization even if several floral buds were noted. In general, as was the case with molecular traits, hybrids showed features that were typical of both parental species. Neither parent prevailed with regard to the transmitted traits and the most striking feature was the inability of hybrids to flower. It should be observed, however, that flowering may only have been delayed, since *M. arborea* flowers about 2 years after sowing and hybrids were transferred into soil only just 2 years ago to date.

This is the first instance of successful symmetric somatic hybridization in *Medicago* between sexually incompatible species. An explanation for the difficulties in obtaining somatic hybrids in the genus *Medicago* was proposed by Arcioni et al. (1995) who, after repeated experiments on somatic hybridization involving various *Medicago* species and explants, concluded that in this genus (unlike other species, Maliga et al. 1977) it is not possible to complement the protoplast mitotic activity of one parent with the regeneration ability of the other. Therefore, in order to obtain somatic hybrid plants both parental protoplasts should be able to regenerate or, when only one is able to regenerate, it should be the one with the greater mitotic activity and plating efficiency; otherwise, the hybrid calli will resemble the non-dividing parents and will not be able to regenerate. This kind of "tissue culture influence" was also observed for the morphological and molecular traits of the somatic hybrid plants. In fact, in a previous hybridization experiment where *M. sativa* contributed the actively dividing and regenerative protoplasts and *M. coerulea* the dormant, non-regenerative ones, most of the *M. coerulea*-specific RFLP fragments were lost by the hybrids which showed a morphological resemblance to *M. sativa* (Pupilli et al. 1995). This is implicitly confirmed by the results of the present paper, where the parents differed neither in mitotic activity nor in regeneration ability, and the hybrids appeared intermediate between *M. sativa* and *M. arborea* for morphological, molecular and cytological features.

The primary aim of the present work was to exploit the possibility of obtaining somatic hybrid plants between cross-incompatible *Medicago* species so as to enrich the alfalfa genome with useful genes such as those for adaptation to stress conditions. Somatic hybrids between genetically distant *Medicago* spp. have now been obtained and traits of interest, such as the low number of stomata, presumably a key factor in the adaptation potential of *M. arborea* to dry environments, have been successfully transferred.

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