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Production of interspecific somatic hybrid plants in the genus *Medicago* through protoplast fusion*

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Summary. Symmetric somatic hybrid plants have been produced by electrofusion of leaf protoplasts of *Medicago sativa* and callus protoplasts of *Medicago coerulea*. The selection of hybrid individuals has been performed at the cellular level by recording the positions of single heterocaryons immobilized in a semisolid culture medium. The hybrid nature of the heterokaryons was assessed in fluorescent light on the basis of their color. Hybrid minicalli were picked up manually and grown first on propagating, and then on regenerating, media. Six putative hybrid calli were selected and two of them regenerated several plants. The hybrid nature of the regenerants was confirmed by cytological and isozyme analysis. Among the several morphological traits taken into account for the characterization of somatic hybrid plants, some were intermediate, some lower, and some higher, with respect to the parents. The somatic hybrid plants were fertile and set seed. The production of somatic hybrid plants in the genus *Medicago* is discussed in relation to the regenerating capability of parental protoplasts.

Key words: *M. sativa* – *M. coerulea* – Protoplast electrofusion – Somatic hybrids – Morphological analysis

Introduction

In the genus *Medicago* interspecific hybridization is a useful method for improving the agronomic value of cultivated species (McEvans 1966; Arcioni et al. 1982). Wild *Medicago* species show several traits of agronomic interest, such as disease, frost and drought resistance, different growth habit, leafiness, high protein content and high seed production (Isaac 1957; McEvans 1966; Mariani et al. 1978). The major barrier to the recovery of hybrid plants is the different ploidy levels of the parental species (Lesins 1962; McLennan et al. 1966; Mariani 1968).

Somatic hybridization overcomes the sexual barriers. The formation of hybrid calli from protoplast fusion has already been reported in the genus *Medicago* (Deak et al. 1988; Damiani et al. 1988; Gilmour et al. 1987, 1989; Walton and Brown 1988; Pupilli et al. 1991). Only in one case (Téoulè 1983), however, were somatic hybrid plants obtained which were able to grow in soil, and here the two species participating in the somatic cross were tetraploid and sexually compatible.

Recently, asymmetric hybrids between tetraploid (*M. sativa* + *M. varia*) and diploid (*M. lupulina* + *M. borealis*) *Medicago* species have been produced (Kuchuk et al. 1990). This paper describes somatic hybrid plants obtained by the electrofusion of mesophyll protoplasts of *M. sativa* (2n=32) and callus protoplasts of *M. coerulea* (2n=16).

Materials and methods

Plant material

Genotype R15 of *M. sativa*, cv. Rangelander (2n=4x=32), selected for regeneration capability through somatic embryogenesis from leaf-derived calli, was obtained from Dr. Larkin (CSIRO, Plant Industry, Canberra) and was propagated by cuttings. A seed-derived plant (C1) of *M. coerulea* (2n=2x=16) (All Union of Plant Industry, Leningrad, USSR), selected for its capacity to regenerate plants from leaf-derived callus, was used and propagated by cuttings. Plantlets were maintained in a con-

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trolled environmental cabinet (216 μE $sec^{-1}m^{-2}$, 20 °C, 12 h photoperiod, 80% relative humidity). *M. sativa* plants were cut when 30 cm tall and leaflets from the regrowth were used for protoplast isolation.

Protoplast isolation

Protoplasts of *M. sativa* genotype R15 were extracted from heart-shaped, fully expanded, hair-free, 5–7 day old leaflets by using the enzyme mixture A as described by Pupilli et al. (1991).

Callus

Fifty seed-derived plants of *M. coerulea* were scored for plant regeneration from leaf calli induced in UM (Uchimiya and Murashige 1974) agar-solidified medium with 2 mgl^{-1} of 2,4-D. A plant (C1), capable of producing calli from which somatic embryos were easily regenerated, was propagated by cuttings and provided the leaves used for callus induction. Four-week old calli were transferred to UM liquid medium (5 g of fresh callus weight in 40 ml of liquid medium in a 250 ml Erlenmeyer flask) and maintained for 4 days on a rotary shaker (22 °C, 20 μE $sec^{-1}m^{-2}$, 150 rpm). The cells were collected by centrifugation and resuspended in an enzyme solution (1:6; v/v) consisting of 10 gl^{-1} Rhozyme HP 150, 10 gl^{-1} Driselase, 1.0 gl^{-1} Cellulase Y-C, 5 mgl^{-1} fluorescein isothiocyanate (FITC) in CPW solution (Frearson et al. 1973) containing 100 gl^{-1} mannitol, pH 5.8. Enzyme digestion was performed on a shaker (50 rev/min) for 7 h at 28 °C in the dark. The enzyme-protoplast mixture was filtered (63 μm stainless steel mesh) and then centrifuged (80 g, 15 min). The pellet was resuspended in CPW solution containing 21% (w/v) sucrose (0.5 ml of pellet in 10 ml of CPW 21S) and centrifuged again (80 g, 15 min). Floating protoplasts were pipetted out, washed twice with the electrofusion solution and resuspended at a density of 3×10^5 protoplasts ml^{-1}.

Protoplasts electrofusion

The electrofusion apparatus and procedure have been previously described (Pupilli et al. 1991). The electrofusion parameters were: alternate current (AC) field strength 210 V cm^{-1}; three direct current (DC) pulses each with a duration of 35 μsec and a voltage of 2,100 V cm^{-1}. After electrofusion, the content of the fusion chamber was transferred to a Petri dish (20 mm) and diluted with 2 ml of mesophyll protoplasts (2.5×10^4 prot. ml^{-1}) suspended in the KM8P culture medium to obtain a plating density of 8×10^4 protoplasts ml^{-1}. For each electrofusion experiment, 3×10^6 protoplasts were electrofused and for each parent 5×10^5 protoplasts were cultured without exposure to the electric field. Two days after fusion, the contents of three dishes were added to a centrifuge tube and cells collected by centrifugation (90 g, 15 min). Six millilitres of supernatant were pipetted off and replaced with 0.8 ml of KM8P plus 1 ml of a 3:1 mixture of double strength KM8P and KM8 cell culture medium (Kao and Michayluk 1975). After cell resuspension, the contents of the tubes were placed in a Petri dish (5 cm) and 1 ml of a warm (30 °C) solution (2% w/v) of "Sea Plaque" agarose (F.M.C. Marine Colloids Division, Rockland, USA) was added. One day after protoplast imbibition the agarose bed of each Petri dish was cut into slices (5 mm wide) and half of them moved to a dish containing 0.5 ml of a 3:1 mixture of KM8P and KM8 culture media solidified with agarose (1%). The slices were fixed to the bottom of the dish with warm (30 °C) agarose (2%) and cultured with 1 ml per Petri dish of a 3:1 KM8P-KM8 mixture. The final density was approximately 1×10^5 prot. ml^{-1} and the cultures were incubated at 28 °C in the dark. Heterokaryons were identified under fluorescent light (red colour for mesophyll protoplasts and yellow for callus FITC-treated protoplasts). Positions (coordinates) of heterokaryons were recorded (100 positions for each experiment). Minicolonies of 30–40 cells were picked-up after 1 month under a stereomicroscope and cultured for 3 weeks on KM8 agarose (0.8%)-solidified medium by placing them in the centre of a Petri dish (2 cm) surrounded by 5–6 calli of R-15 mesophyll protoplasts. The cultures were incubated under fluorescent light, 27 μE $sec^{-1}m^{-2}$, 12 h photoperiod, 23 °C and subcultured weekly.

Plant regeneration

The minicalli were grown for an additional 3 weeks on UM agar-solidified medium (0.8% w/v) with 2 mgl^{-1} of 2,4-D and subcultured every 10 days. The hybrid calli, together with mesophyll protoplast-derived calli, were regenerated according to the procedure of Arcioni et al. (1989). The embryo-like structures which developed on the surface of the hybrid callus were picked up with a small amount of surrounding callus and cultured for 6 weeks on MS (Murashige and Skoog 1962) containing 1 mgl^{-1} of 2-iP, 0.1 mgl^{-1} of IAA, 20 gl^{-1} sucrose, agar 8 gl^{-1}. In this medium secondary embryos were formed which developed into plantlets either in the same medium or in MS hormone-free medium (MSO). Calli and regenerative structures maintained on MS1 were subcultured every week. Plantlets rooted easily in MSO and when 5–10 cm tall were transferred to soil.

Hybrid recognition

Chromosome number was determined according to Piccirilli et al. (1988).

Isozyme analysis for peroxidase was carried out on starch-gel electrophoresis as reported by Arcioni et al. (1980) with some modifications. Proteins were extracted by crushing; for each plant, 200 mg of shoot apex and 100 mg of young rootlets were crushed with three drops of chilled gel buffer consisting of Tris-citric acid (15 mM and 3 mM, respectively), pH 7.8. The running buffer was a solution of 0.3 M boric acid adjusted to pH 7.8 with NaOH. The gels were sliced into three layers 2 mm thick, stained for peroxidase (Shaw and Prasad 1970) and fixed in a solution of 50% ethanol (v/v).

Isoenzyme analysis for esterase followed the procedure reported by Pupilli et al. (1991), but proteins were extracted from 100 milligrams (fresh weight) of leaves, frozen in liquid nitrogen and homogenized in the presence of 0.5 g of sand and 100 μl of 0.2 M Tris-glycine buffer (pH 8.9).

Morphological traits were evaluated for 25 plants each of: (1) *M. sativa* derived from mesophyll protoplasts, (2) cuttings of the *M. coerulea* genotype (C1), and (3) putative somatic hybrid plants grown in the greenhouse for 4 months. The characters considered were: (1) plant habit, by scoring from 1 (prostrate) to 9 (erect); (2) internode length (mm) between the second and the third node of the longest stem measured after the appearance of the fourth node; (3) stipule length (mm) taken from the 2nd, 3rd and 4th node of the central stem. The trifoliate leaf placed on the median portion of the longest stem was scored for (4) leaflet area (mm^2), (5) stomatal characteristics (number per mm^2, length and width expressed in μm), (6) pubescence; the central leaflet of the same trifoliate leaf was scored for (7) length (mm), width (mm) and length/width ratio, (8) indentation (number of teeth per leaflet and tooth depth in mm). For each of these traits with the exception of plant habit, an analysis of variance was carried out. Flower colors were evaluated according to the Munsell Color Charts for Plant Tissues (Kollmorgen Corporation, Baltimore, Maryland).

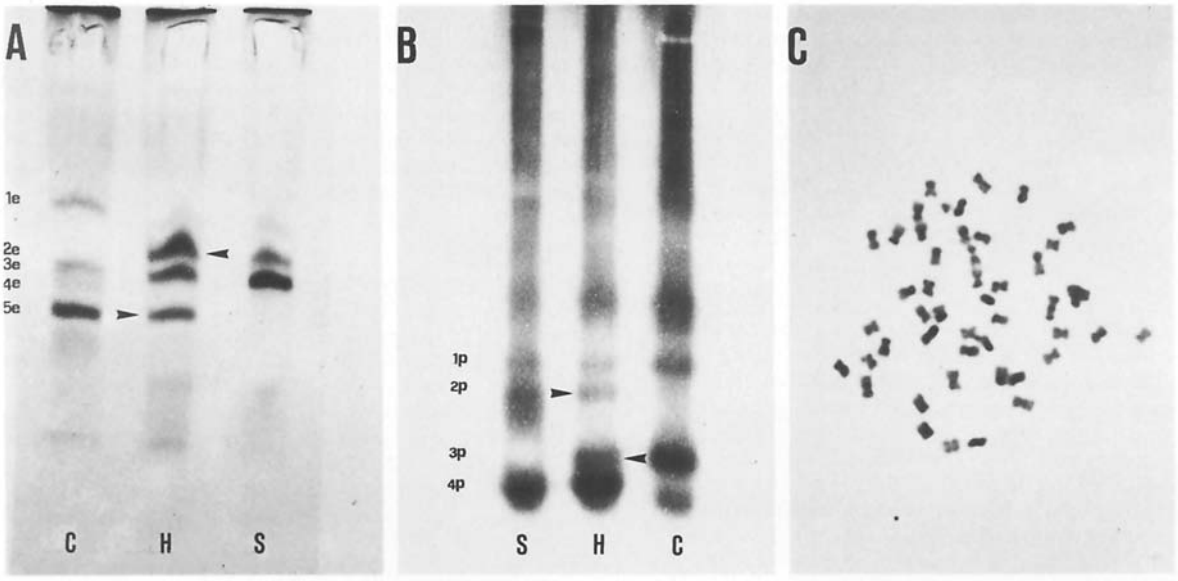

Fig. 1. **A** Esterase patterns of *M. sativa* (*S*), *M. coerulea* (*C*) and somatic hybrid (*H*). **B** Peroxidase patterns of *M. sativa* (*S*), *M. coerulea* (*C*) and somatic hybrid (*H*). The characteristic bands of each parent expressed in the hybrid are indicated by the *arrows*. **C** Metaphase chromosomes of the somatic hybrid

Results

Protoplast culture

Mesophyll protoplasts of the R15 genotype showed the first mitotic divisions 8–10 days after plating. Division frequency and plating efficiency were 72% and 47%, respectively. In comparison with liquid culture, the agarose bed nearly doubled protoplast division and plating efficiency. A large proportion of protoplasts produced proembryo-like structures consisting of a cluster of densely, and very cytoplasmic, compacted cells. These grew into globular embryos and most of them developed through globular, torpedo and cotyledonary stages if, when 1 mm in diameter, they were transferred to KM8 agar-solidified medium and maintained under fluorescent light. If the embryo-like structures were maintained in agarose, only a few entered the next stages of embryogenesis while most formed calli that later differentiated into embryos when cultured in succession on KM8, UM1 and MS1 solid media. A very low number of the primary embryos (1–2%) developed directly into plants. Usually, when the embryos reached the early cotyledonary stage they generated an abnormal structure, sometimes with a well developed root. This produced a small amount of callus and several embryos, generated directly from almost all parts of the primary embryo. which were able to grow into complete plantlets.

In *M. coerulea*, leaf-derived calli were induced on UM1 and after 3–4 weeks of culture showed localised greening. These calli, as previously noted (Arcioni et al. 1982), developed into embryoids after transfer to hormone-free MS medium. Callus protoplasts showed a low division frequency (2%) and rare minicolonies were formed. In contrast, protoplasts isolated from cell suspensions showed a plating efficiency of around 30%, but calli derived from them did not regenerate plants.

Protoplast fusion and selection of somatic hybrids

Two hours after fusion, in three separate experiments involving 5×10^6 protoplasts, the number of heterokaryons recorded, was equal to 6–7% of the total protoplast population. The heterokaryon viability decreased with time in culture: 2 days after fusion 80% of hybrid cells were alive while 2 days later the percentage decreased to 60%. Most of the dead heterokaryons were polyfusion products. Approximately 10–12% of the scored heterokaryons entered division by day 7 of culture and almost half of them also underwent the second and the third division. Microcolonies reached the 8–10 cell stage in 4–6 days from the first mitotic division. Hybrid cells started division 2–3 days before *M. sativa*, while cells of *M. coerulea* divided only occasionally; heterokaryons did not evolve into embryo-like structures, as did the *M. sativa* protoplasts, but formed only callus. After 4 weeks, putative hybrid minicolonies were picked up, transferred to KM8 agar solidified medium and incubated under fluorescent light. In total 330 heterokaryon coordinates were recorded; ten hybrid calli were picked up, six of these remained alive and continued to grow and only two developed abnormal shoot-like structures when placed on MS1 medium. As these structures did not develop into plants, they were de-differentiated on SH agar-solidified medium with 15 mM ammonium,

100 mM proline and 3% maltose (Stuart and Strickland 1984). Here they produced embryos which readily developed into plants. These were maintained 4 weeks in MSO agar medium prior to transfer into soil. In total we recovered about 50 plants which set seeds either after selfing, or polycross, or backcross mating with both parents.

Enzyme analysis

The nuclear constitution of regenerated plants (five putative somatic hybrids were considered) was demonstrated by esterase and peroxidase isozyme profiles. Classification of plants as either somatic hybrids or parental types was possible because the genotypes of *M. sativa* and *M. coerulea* exhibited different patterns for both isozymes tested (Fig. 1 A, B). As far as esterase is concerned (Fig. 1 A), in *M. coerulea* we found three bands (1e, 4e, 5e) not present in the genotype R15 of *M. sativa*. This last parent had only one specific band (2e). The two species also differed in the level of staining of band 3e which was more intense in *M. sativa*. Plants regenerated from selected hybrid calli displayed bands specific to *M. sativa* (2e) and *M. coerulea* (5e). Some bands (1e and 4e) typical of *M. coerulea* were not expressed.

The peroxidase zymogram from parental and hybrid plants is shown in Fig. 1 B. The parents showed two identical bands (1p and 4p) and two specific bands (3p and 2p for *M. coerulea* and *M. sativa*, respectively). All parental bands were clearly present in the somatic hybrids. Isozyme analysis of the somatic hybrid revealed that peroxidase genes of both parental nuclei were expressed. In the esterase zymogram of the hybrids, the absence of some parental bands is consistent with the reports of other authors (Wetter 1977; Wetter and Kao 1976). In the hybrid, two-thirds of the genome is from *M. sativa* and one-third from *M. coerulea*, and only the faint bands (1e and 4e) of *M. coerulea* were not expressed.

Chromosome analysis

Parental chromosomes were similar in size and morphology (Fig. 1 C); thus the study was restricted to chromosome counts of 14 putative hybrid plants. The somatic hybrid plants had a chromosome number ranging from 45 to 50; the most frequent chromosome numbers were 48 (four plants) and 46 (five plants), but regenerants with 50 (two plants), 47 (one plant) and 45 (two plants) were also observed. As expected, the hybrid plants exhibited a chromosome number that was a combination of the parental genomes, giving rise to an allohexaploid ($2n = 6x = 48$).

Morphology of regenerated plants

The morphological characteristics of protoplast- and callus-derived plants of *M. sativa* (R-15), *M. coerulea* (C1) and of somatic hybrids are reported in Table 1 and Figs. 2 A–C. In vitro the growth of hybrid plants was slower than the parents, but after transfer to the greenhouse somatic hybrids appeared vigorous with a more erect growth habit than parental plants of comparable age (Fig. 2 B). The morphology of leaves of hybrid plants was intermediate with respect to the parents (Fig. 2 A). Leaflets of *M. sativa* were oval in shape, with a length slightly larger than width, while those of *M. coerulea* had a stretched oval shape with a length twice the width. Leaflets of somatic hybrid plants were heart-shaped with almost the same values for length and width (the length/width ratios were 1.33, 1.07 and 2.03 for *M. sativa*, somatic hybrids, and *M. coerulea*, respectively). The internode length and leaf area of somatic hybrids were higher than those of *M. coerulea* and did not differ significantly from *M. sativa*. The number of hairs per mm^2 on the abaxial leaf surface, mainly along the mid-veins, were much higher (almost double) in the hybrid plants compared to *M. sativa* but were similar to *M. coerulea*. The stipules in the hybrid plants were two and three times longer than in *M. sativa* and *M. coerulea*, respectively (Fig. 2 C). The leaf margin was toothed in both parents but the number of teeth per leaflet was much higher in *M. coerulea* (16.45) than in *M. sativa* (8.78); the hybrid plants showed an abundant indentation not different from *M. coerulea* but much (4–6 times) deeper than in both parents. *M. coerulea* was characterized by a higher number of stomata per unit area (146.3), but of reduced size (almost half) with respect to *M. sativa*. The somatic hybrid plants showed a lower stomata density, but of larger size, with respect to both parents. The flowers of parents and hybrids were red purple (5rp) but differed for color intensity which was 5/4, 7/2 and 6/2 for *M. coerulea*, *M. sativa* and somatic hybrids, respectively.

Table 1. Morphological traits evaluated in *M. sativa* (1), somatic hybrids (2), and *M. coerulea* (3). The values followed by the same letter are not significantly different for $P \leq 0.05$.

Character	1	2	3
Growth habit	6–7 nd	7–8 nd	2–3 nd
Internode length (mm)	40.65 a	35.41 a	21.33 b
Leaflet length (mm)	14.50 a	13.29 a	11.43 b
Leaflet width (mm)	10.60 b	11.52 a	5.67 c
Leaflet length/width	1.33 b	1.07 c	2.03 a
Leaflet area (mm^2)	93.69 a	95.48 a	38.57 b
Indentation (number/leaflet)	8.78 b	16.04 a	16.45 a
Indentation depth (mm)	0.44 b	1.63 a	0.29 b
Pubescence (number/mm^2)	21.33 b	36.00 a	34.67 a
Stomata (number/mm^2)	67 b	60 c	146.30 a
Stomata length (μm)	25.75 b	32.00 a	15.55 c
Stomata width (μm)	16.00 b	18.50 a	9.75 c
Stipule length (mm)	5.83 b	9.00 a	3.86 c

nd, not determined

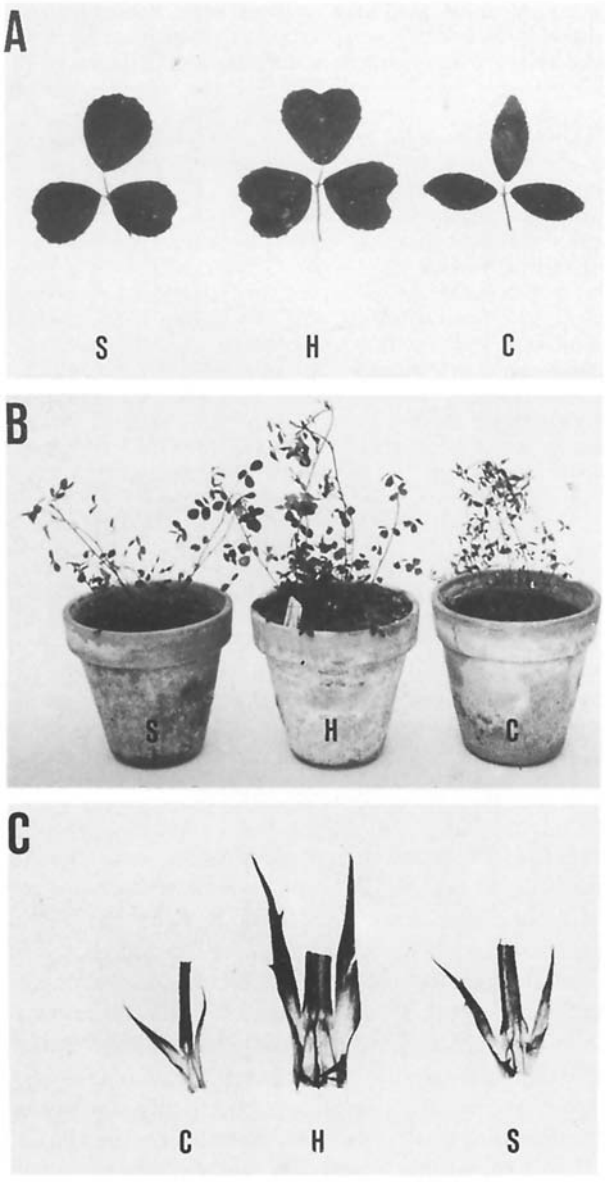

Fig. 2. **A** Leaflet morphology of *M. sativa* (*S*), *M. coerulea* (*C*) and somatic hybrid (*H*). **B** Regenerated plants of *M. sativa* (*S*), *M. coerulea* (*C*) and somatic hybrid (*H*). **C** Stipule length of *M. sativa* (*S*), *M. coerulea* (*C*) and somatic hybrid (*H*)

Discussion

This study, for the first time, reports the production in the genus *Medicago* of somatic hybrid plants following fusion between mesophyll and callus protoplasts of two species of different ploidy level. In the genus *Medicago*, hybrid plants derived from symmetric hybridization have so-far been reported only by Téoulè (1983) who fused mesophyll protoplasts of both parents. On the other hand, when one fusion partner was represented by cell-suspension or callus protoplasts, even if induced from a genotype selected for high regeneration capacity, the hybrid calli failed to regenerate plants (Damiani et al. 1988; Deak et al. 1988; Gilmour et al. 1987, 1989; Pupilli et al. 1991). Cell-suspension protoplasts have a higher division frequency and plating efficiency than mesophyll protoplasts and heterokaryons and hybrid calli reflect the physiological activity of the parental cell-suspension protoplasts, which is the major cytoplasm donor (Gleba and Hoffman 1978).

In experiments carried out in our laboratory, it has been observed that the more responsive the cell-suspension protoplasts are in vitro, the lower is their plant regeneration capacity. Thus, cell suspensions induced from leaf-derived calli of two genotypes (P1 and R15) of *M. sativa* selected for morphogenetic response, released protoplasts with high plating efficiency (60–70%), but none of 400 protoplast-derived calli tested became embryogenic. By contrast, mesophyll protoplast-derived calli showed a regeneration frequency of around 90% (unpublished data). The situation found when cultivating callus protoplasts is similar, but less negative, because the in-vitro responsiveness is lower, and morphogenesis higher, than in cell-suspension protoplasts.

In the genus *Medicago* it seems that regeneration from hybrid calli can be achieved not only when both parental protoplasts are regenerable, but also when protoplasts of only one parent are morphogenetic and with a plating efficiency much higher than that of the other parent. Under these conditions heterokaryons follow the developmental pathway of the morphogenetic parent. In this study *M. coerulea* protoplasts divided occasionally, while those of *M. sativa* showed both high plating efficiency and morphogenetic capacity and heterokaryons behaved as the more active protoplasts in vitro.

M. coerulea was initially described as a separate species to *M. sativa*; more recently *M. coerulea* was classified as a subspecies and identified as *M. sativa* spp. *coerulea* (Lessing ex Ledebour) Schmalhausen (Lesins and Lesins 1979). Within the *Medicago sativa-falcata-coerulea* complex, no barriers impede sexual crosses at the same ploidy level, whereas, if the parents show different chromosome numbers, their crossability is seriously restricted. Even if hybridization between *M. sativa* and *M. coerulea* can be achieved sexually, the strategy employed in the present study can be applied for transferring, into cultivated alfalfa, the germplasm of those *Medicago* species, sexually incompatible with *M. sativa*, which carry traits of economic importance. The selection method used can, therefore, be applied to any fusion experiment without the need for particular mutants, which often carry undesirable traits.

On the basis of our results, the production of somatic hybrid plants between alfalfa and other species of *Medicago* can be attempted by fusing morphogenetic and highly dividing protoplasts of a selected genotype of *M. sativa* with protoplasts of other *Medicago* species, pre-

pared from several tissues (with the exclusion of cell suspensions), and characterized by a low plating efficiency. These requirements can be easily satisfied because in all alfalfa cultivars it should be possible to find a responsive genotype if enough plants are screened. The wild species are only required to release protoplasts from different plant organs and calli and should be used even if cell divisions are rare (our unpublished data). In the genus *Medicago*, fertility limitations related to polyploidization subsequent to protoplast fusion should not be a problem: some allotetraploid (2n=32) and alloautoexaploid (2n=48) species have already been found in nature (Lesins and Lesins 1979).

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