PLANT REGENERATION FROM PROTOPLASTS OF ALFALFA (*Medicago sativa*) VIA SOMATIC EMBRYOGENESIS

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ABSTRACT: Alfalfa is one of the most frequently studied species from the production of tissue culture-derived embryos point of view. In this study, five alfalfa cultivars were analyzed with reference to their ability to regenerate plants from protoplast cultures via somatic embryogenesis. Plant regeneration from leaf-derived protoplasts isolated from the cultivar Rangelander was achieved using a protocol defined for alfalfa cell suspension-derived embryogenesis. Because of its high efficiency, this procedure is recommended for protoplast electroporation-mediated genetic transformation of alfalfa.
Key words: plant biotechnology, *in vitro* culture, morphogenesis, forage legume

REGENERAÇÃO DE PLANTAS A PARTIR DE PROTOPLASTOS DE ALFAFA (*Medicago sativa*) VIA EMBRIOGÊNESE SOMÁTICA

RESUMO: A alfafa é uma das espécies mais freqüentemente estudadas do ponto de vista da produção de embriões somáticos derivados da cultura de tecidos. Neste trabalho, cinco cultivares de alfafa foram analisados com referência à capacidade de regenerar plantas a partir de culturas de protoplastos via embriogênese somática. Regeneração de plantas a partir de protoplastos isolados de folhas da cultivar Rangelander foi obtida usando-se um protocolo definido para embriogênese somática derivada de suspensões celulares de alfafa. Em função da sua alta eficiência, recomenda-se o uso deste procedimento para transformação genética de alfafa mediada por eletroporação de protoplastos.
Palavras-chave: biotecnologia de plantas, cultura *in vitro*, morfogênese, leguminosa forrageira

INTRODUCTION

Alfalfa is a warm season perennial legume planted worldwide. Originated in the Middle-East near Iran, is now found in nearly all continents. It represents one of the oldest forage crops. Depending upon the cultivar it withstands a wide variety of climates and is highly drought resistant through dormancy for as much as two years. Having high minerals contents, vitamins and protein, alfalfa is one of the most nutritious crops that can be utilized as forage. Alfalfa is also used for silage, hay, green chop, erosion control, honey production, rotational grazing, and in some forms, for human consumption. It is a high-quality forage plant for all livestock types. Alfalfa is also a good nitrogen soil fixer, by symbiosis with bacteria of the genum *Rhizobium* (www.farmseeds.com/forages/alfalfa).

In Brazil, alfalfa cropping began in the South (Coelho Jr., 1994), but now it is possible to grow alfalfa in the Southeastern and Northeastern regions of the country. 'Crioula' is the most accepted variety, as a result of its adaptability and productivity (28 t DM per ha per year). It is used feed for horses, cattle, dairy cattle, sheep, and also ostriches (Haddad & Castro, 1999).

Alfalfa breeding through genetic manipulation approaches is a current methodology to obtain pest and herbicide resistances (D'Halluin et al., 1990). It is also directed towards combining genomes of *Medicago* through somatic hybridization such as *M. sativa* + *M. borealis* L. (Gilmour et al., 1989) *M. sativa* + *M. falcata* L. (Mendis et al., 1991), *M. sativa* + *M. coerulea* L. (Pupilli et al., 1992), and *M. sativa* + *M. arborea* (Nenz et al., 1996).

Alfalfa is also one of the most frequently studied crops from the point of view of tissue culture-derived embryo production. It is also considered the species with the most advanced synthetic seed system, although its use for commercial propagation purposes is still under evaluation (Piccioni et al., 1997). Moreover, significant progress has been made using genetic engineering methods for improving its nutritional value (Schroeder et al., 1991), to enhance tolerance to abiotic stress (McKersei et al., 1993), and to use alfalfa as a source of value-added products.

Technologies are available to introduce genes into alfalfa via genetic transformation, but commercially important cultivars cannot yet be engineered. Regenerability capacity is the primary constraint to successfully transform elite genotypes. Preliminary studies indicate that alfalfa *in vitro* response is strongly genotype dependent (Bingham et al., 1975; Atanassov & Brown, 1984). This feature was also reported by Myers et al. (1989) working on *Trifolium pratense* L. Also, as a rule, growth regulators have a critical importance in inducing somatic embryogenesis in legumes.

Atanassov & Brown (1984) reported the induction of callus from cotyledon, hypocotyl and leaf-derived alfalfa explants on B_5h medium, which consists of B_5 medium (Gamborg et al., 1968) modified to contain 1 mg L^{-1} 2,4-D (2,4-dichlorophenoxyacetic acid); 0.2 mg L^{-1} kinetin; 30,000 mg L^{-1} sucrose; 3,000 mg L^{-1} KNO_3; 895 mg L^{-1} $CaCl_2$; 0,800 mg L^{-1} L-glutamine; 500 mg L^{-1} $MgSO_4 \cdot 7\ H_2O$; 100 mg L^{-1} serine; 10 mg L^{-1} L-glutathione; 1.0 mg L^{-1} adenine and 9 g L^{-1} Bacto agar. Primary calli, three weeks after subculturing, were used to establish cell suspension cultures that were maintained by subculturing 2-5 mL of the topmost layer of settled cell clusters into 45 mL of fresh B_5h medium every seven days. Under these conditions, cells had a characteristic growth curve, and various stages of differentiation were observed in the newly established cell suspensions. All of the 13 tested varieties produced embryos on the B_5h $\rightarrow$ SHb $\rightarrow$ BOi2Y media sequence: a treatment on solid (8 g L^{-1} Bacto agar) SHb medium, which consisted of Shenk & Hildebrandt (1972) inorganic and organic components plus 11 mg L^{-1} 2,4-D and 1mg L^{-1} kinetin, followed by culture on BOi2Y that consisted of modified Blaydes (Bingham et al., 1975) inorganic and organic constituents plus 100 mg L^{-1} meso-inositol, 2 g L^{-1} yeast extract and 10 g L^{-1} Bacto agar. Large green embryos were formed and transferred after 14 days to hormone-free SH or B_5 media, continued development and begin rooting after 14 days. The responses of 'Rangelander', 'Regen S' and 'Rambler' were 100, 67 and 53%, respectively, of individual calli forming embryos. The last two produced somatic embryos from which plants could be regenerated and successfully acclimatized. Plant regeneration was achieved.

Based on previous studies on forage plants, namely *Trifolium rubens*, it is possible to regenerate plants from leaf tissues and cell suspension-derived protoplasts (Grosser & Collins, 1984). Therefore the protocol of Atanassov & Brown (1984) was used to obtain somatic embryos and, subsequently, plants from *M. sativa* 'Rangelander' protoplasts. This system should allow microparticle bombardment and protoplast electroporation, the principal mechanisms for direct gene delivery into legume plant cells (Aragão et al., 1999; Potrykus, 1991; Quecini et al., 2002). Genetic engineering methods are very useful for the transference of disease resistance genes into alfalfa genome in Brazil, namely to control the fungi *Rhizoctonia solani* and *Colletotrichum trifolii.*

The present paper describes plant regeneration from protoplasts isolated from five varieties of *M. sativa* L. Using histological techniques, the characteristics of the protoplast-derived somatic embryos were investigated. Phenotypic and molecular analyses were also carried out between the donors and corresponding regenerated plantlets. The RAPD technique was used for genomic DNA fingerprinting.

MATERIAL AND METHODS

Plant material

Seeds of four alfalfa cultivars Crioula, Alfa 200, Valley Plus and Semit 921, cultivated in the subtropical regions of South America, and of the cultivar Rangelander (CSIRO, Division of Plant Industry, Australia), were disinfected by immersion in a 1.5% (v/v) NaOCl solution for 6 min, followed by 4 rinses in sterilized water. Seeds were scarified and placed on half-strength MS basal medium (Murashige & Skoog, 1962) to obtain aseptic material. Because of the small number of seeds available for cultivar Rangelander, nodal cuttings were used to produce whole plantlets within 6 to 7 weeks from the beginning of each subculture. Propagation was done through axillary branching.

Protoplast isolation and culture

For protoplast isolation, 250 mg of cotyledonary tissues of 'Crioula', 'Alfa 200', 'Valley Plus' and 'Semit 921', or leaf-derived explants excised from all five genotypes were used. For plasmolysis, tissues were maintained in a CPW-13 solution (Frearson et al., 1973) for 1 h and then submitted to an enzyme-digestion (2.0% Cellulase R10 and 0.5% Macerozyme R10, Yakult) for 17 h under shaking (35 rpm) in the dark. Protoplast washing, counting and viability estimation were performed according to Dornelas & Vieira (1993), described in detail by Monteiro (2000).

Protoplasts were cultured at three densities, i.e. 0.5, 1.0 and 1.5 x 10^5 protoplasts per mL of agarose-solidified (0.6%, Sigma type VII) K8P medium (Kao, 1977; modified by Gilmour et al., 1989) for 5 d in the dark. Cultures were then kept at 25 ± 2°C, with a 16 h light regime, and 25 $\mu M\ m^{-2}\ s^{-1}$ light radiation. A mixture of K8P: K8 (Kao, 1977; modified by Gilmour et al., 1989) was used for a progressive replacement of one third of the bathing medium after 7 (2:1), 14 (1:1) and 21 (0:1) days. At this time, plating efficiencies were calculated as the number of colonies in relation to the initial number of plated protoplasts by microscopic evaluation. Twenty fields (20 X objective) and 3 plates were used for each

combination, i.e., genotype vs. protoplast source vs. plating density. Data were analyzed by current statistical analysis methods.

After 28 d, resulting microcalli were transferred to agar-solidified (0.6% Sigma) B_5h medium (Gamborg et al., 1968; modified by Atanassov & Brown, 1984) for 28 d. Cultures were then transferred to agar-solidified SHb medium (Walker et al., 1979) for 21 d, and BOi2Y medium (Bingham et al., 1975) for 28 d. Half-strength MS medium was used for embryo germination. Regenerated plantlets of 'Rangelander' were transferred to a greenhouse and acclimated.

Anatomical studies

Embryo-like structures derived from 'Semit 921' and 'Rangelander' protoplasts were analyzed by histological procedures. Samples were fixed in Karnovsky solution (Karnovsky, 1965), dehydrated through a series of ethanol treatments, and embedded in glycol metacrylate resin (Reichert-Jung). Longitudinal sections (5 µm) were stained in 0.05% (w/v) toluidin blue O (Sakai, 1973). A Nikon AFX-DX microscope was used for photo documentation.

DNA fingerprinting protocols

Molecular analysis based on Random amplified polymorphic DNA (RAPD) markers was carried out to verify genetic identity, comparing the four explant-donors and the *in vitro* derived plantlets of 'Rangelander'. Total genomic DNA was extracted from leaf tissues (250 mg) using a modified CTAB method as described by Vieira et al. (1997). DNA samples were diluted in TE buffer and submitted to electrophoresis (3 V cm^{-1}) in 0.8% agarose gels (w/v). DNA was stained by gel immersion into ethidium bromide solution (10 µg mL^{-1}) for 30 min. To quantify the DNA, the fluorescence intensities of the samples were compared to those of l DNA standards.

RAPD reactions were performed in a final volume of 25 µL in PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl) containing 3.0 mM $MgCl_2$, 200 µM primers (Operon Technologies, Kit A), 0.2 mM of each dNTP, and 1.5 units of Taq DNA polymerase (Gibco BRL). Ten, 20 and 30 ng of template DNA were used. Reactions were covered with mineral oil and submitted to the following PCR program (PCR Express Hybaid machine): preliminary DNA denaturation for 5 min at 94°C, followed by 45 cycles consisting of denaturation (1 min, 94°C), primer annealing (1 min, 35°C), and extension (2 min, 72°C). A final extension for 6 min at 72°C was included. The RAPD products were separated by electrophoresis (3 V cm^{-1}) in 1.4% agarose gels, which run with 1 x TBE buffer. Photo documentation was performed under UV light using a video imaging system (Bio Rad).

RAPD data were analyzed using locus-to-locus gel readings and the rates of *in vitro* DNA polymorphism were calculated. Only polymorphisms present in all DNA concentrations were included in the analysis. The formula of Dice (1945) was used to calculate the similarity between each donor and its embryo-derived plantlets.

RESULTS AND DISCUSSION

In vitro procedures

The enzymatic treatment was efficient for liberating cotyledon or leaf-derived protoplasts, and high viabilities were observed for all genotypes (Table 1). Cultured protoplasts grew very fast. After 24 h the cell-walls were regenerated, and first mitotic divisions were subsequently observed. Cell colonies were observed after 7 d (Figure 1 A-D). Microcalli were prominent in all cultures after 21 d, and cotyledon-derived protoplasts cultured at 0.5 x 10^5 led to superior plating efficiencies. 'Crioula' cotyledon-derived protoplast cultures developed calli at a plating efficiency ratio of 17.5% while from leaf-derived plates, the

Table 1 - Production, viability and plating efficiency (PE) in protoplast cultures of *M. sativa* obtained from cotyledon (C) explants at density of 0.5 x 10^5, and leaf explants (L), at density of 1.0 x 10^5, according to the cultivar.

Cultivar	Explant	Protoplast production[1]	Viability (%)[2]	PE[3]	Number of colonies 21 d after plating[4]
Crioula	C	1.13 x 10^6 ± 0.20	88.32 ± 7.26	0.175 ± 0.08	3,952.5 ± 1,820.8
	L	1.14 x 10^6 ± 0.16	82.24 ± 4.26	0.039 ± 5.03	1,770.0 ± 226.4
Alfa 200	C	1.06 x 10^6 ± 0.16	80.66 ± 10.16	0.137 ± 0.03	3,097.5 ± 843.7
	L	1.27 x 10^6 ± 0.19	78.29 ± 4.56	0.027 ± 3.51	1,230.0 ± 158.0
Valley Plus	C	1.03 x 10^6 ± 0.11	84.77 ± 9.03	0.082 ± 0.01	1,845.0 ± 250.5
	L	1.11 x 10^6 ± 0.18	80.72 ± 6.18	0.031 ± 6.08	1,395.0 ± 273.7
Semit 921	C	1.18 x 106 ± 0.16	85.21 ± 3.50	0.075 ± 3.60	1,687.5 ± 81.1
	L	1.06 x 10^6 ± 0.23	85.61 ± 3.48	0.033 ± 5.50	1,515.0 ± 274.8
Rangelander	L	1.43 x 10^6 ± 0.34	85.53 ± 4.13	0.060 ± 8.71	2,700.0 ± 392.3

[1]Mean of 10 experiments ± standard error (production from 250 mg tissue per 5 mL^{-1} enzyme solution)
[2]Mean of 20-microscope fields ± standard error (freshly isolated protoplasts)
[3]Mean of 3 plates ± standard error. PE = Number of colonies/ Number of plating protoplasts
[4]Mean of 3 plates ± standard error (agar droplet-based estimative)

ratio was only 2.2% (Figure 2). Differences between densities, protoplast sources, and cultivars as well as the interaction between densities *versus* protoplast sources (explants) were all significant (Figure 2) ($P < 0.05$). At all densities, the response of cotyledon-derived cultures was statistically different in comparison to leaf-derived cultures.

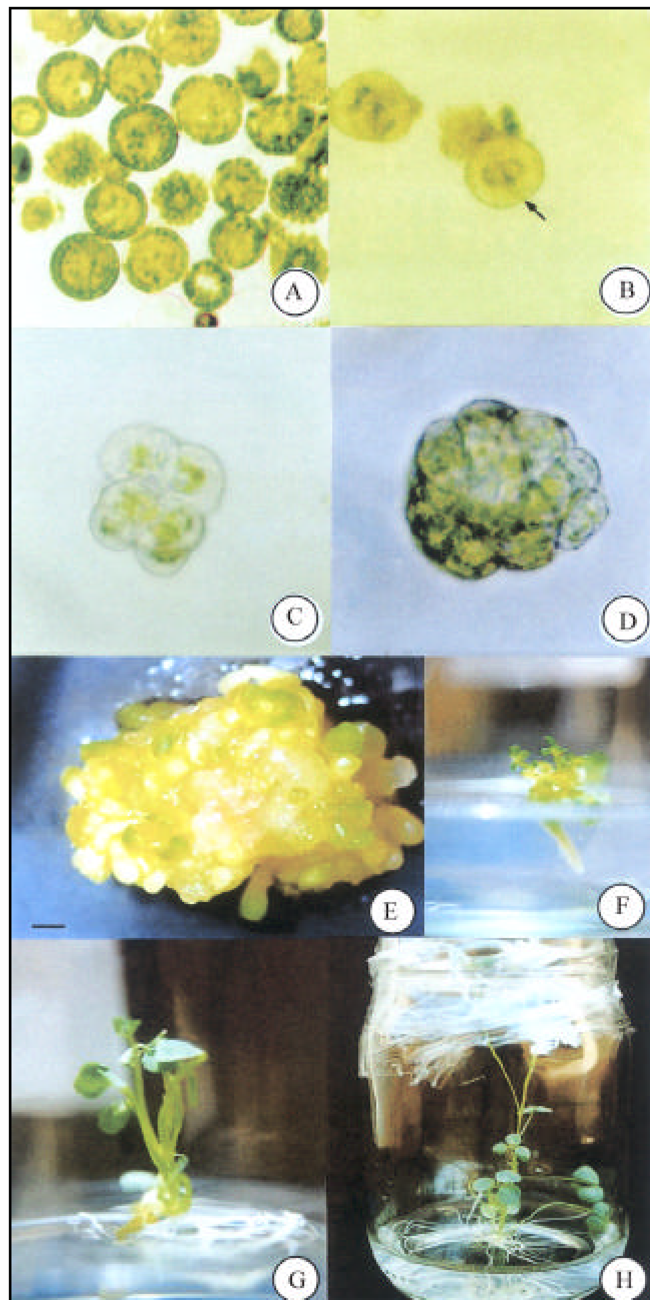

Figure 1 - Multicellular colony development in *Medicago sativa* cultivar Semit 921 cotyledon-derived protoplast cultures: A. Freshly isolated protoplasts (400 x). B. First mitotic division 72 h after isolation (400 x). C. Developed divisions (400 x). D. Microcolonies 7 days after isolation (160 x). E-F. Callus presenting embryoid-like structures derived from leaf protoplasts of *Medicago sativa* 'Rangelander', and embryo germination (bar = 870 µm). G-H. Whole plantlets after 60 and 90 days on half-strength MS medium.

Leaf-derived protoplasts isolated from 'Rangelander' were very competent for embryogenic callus formation when cultivated at 1.0 x 10^5 cells per mL of K8P protoplast culture medium. Approximately 100 calli developing from the five cultivars were transferred to solid medium (SHb) containing a high auxin/low cytokinin ratio i.e. 11 mg L^{-1} 2,4-D and 1mg L^{-1} kinetin. After the transfer to BOi2Y medium, only 'Rangelander' cultures gave rise to somatic embryos. Although very promising at the cellular stage, no differentiation was observed on the 'Crioula', 'Alfa 200' and 'Valley Plus' plates.

To induce embryo formation on the 'Crioula', 'Alfa 200' and 'Valley Plus' cultures (data not shown), colonies were transferred to UM (Uchimiya & Murashige, 1974) containing 2,4-D at 2, 4, 6, 8 and 10 mg L^{-1}, and also to B1INK (Parrot et al., 1995) media, but all attempts failed to induce embryos. As previously suggested, high influence of the genotype on the *in vitro* response of alfalfa, especially for somatic embryogenesis was observed (see Bingham et al., 1975, Kao & Michayluk, 1980, Chen et al., 1987; Atanassov & Brown, 1984; Du et al., 1994).

On average, 20.3 ± 12.7 embryoids per callus occurred in the 'Rangelander' cultures (Figure 1E). In addition secondary embryoids were easily proliferated. After 20 d, cotyledonary embryos were excised and transferred to half-strength MS (Figure 1F). Whole plantlets were formed in the subsequent 90 d (Figure 1G-H). Acclimatization to greenhouse conditions was successful.

Electroporation is an efficient technique to introduce exogenous DNA into plant cells (Potrykus, 1991). In *Stylosanthes guianensis*, a tropical forage legume, direct DNA uptake using an electroporation-mediated method (3.2 x 10^7 electroporated protoplasts produced 50 regenerants, from which 18 were transgenic) was superior to a biolistic approach (Quecini, 1999; Quecini et al., 2002). The protocol described herein could be used for genetic transformation of alfalfa, and somatic hybridization experiments involving *Medicago* species.

Anatomical studies

Embryo-like structures were observed after 60 d in the 'Rangelander' cultures (Figure 3a). Serial longitudinal sections revealed that these structures were bipolar, with a defined protoderm, ground meristem and procambium, typical of a heart stage embryo (Figure 3c). The somatic embryogenesis was as asynchronous as described for other legumes like *Stylosanthes scabra* and *Glycine max* by Dornelas et al. (1992) and Fernando et al. (2002), respectively. Some alfalfa embryos presented new embryoids of epidermal origin, close to their hypocotyledonary region.

On the Semit 921 plates, embryo-like structures, similar to those described by Téoulé & Dattée (1987),

were observed (Figure 3b). However, the histological analysis showed that they were friable calli with typical parenchyma cells. Root meristems were identified, characterizing the rhizogenesis process (Figure 3d).

RAPD analysis

Template DNA concentrations of 20 and 30 ng produced clear PCR profiles. RAPD fingerprints were always identical to that of the explant-donor plants, but in a leaf-protoplast derived plant of 'Rangelander', primers A01, A04 and A12 detected differences, i.e. among 19 loci, 6 (31.5%) were polymorphic (Figure 4). The similarity was 81.25% according to the Dice coefficient. Although *in vitro*-induced polymorphism at DNA level was observed, no morphological or even reproductive features of the adult plant were anomalous.

Several authors have used molecular markers to monitor genetic stability in *in vitro* culture-derived plants,

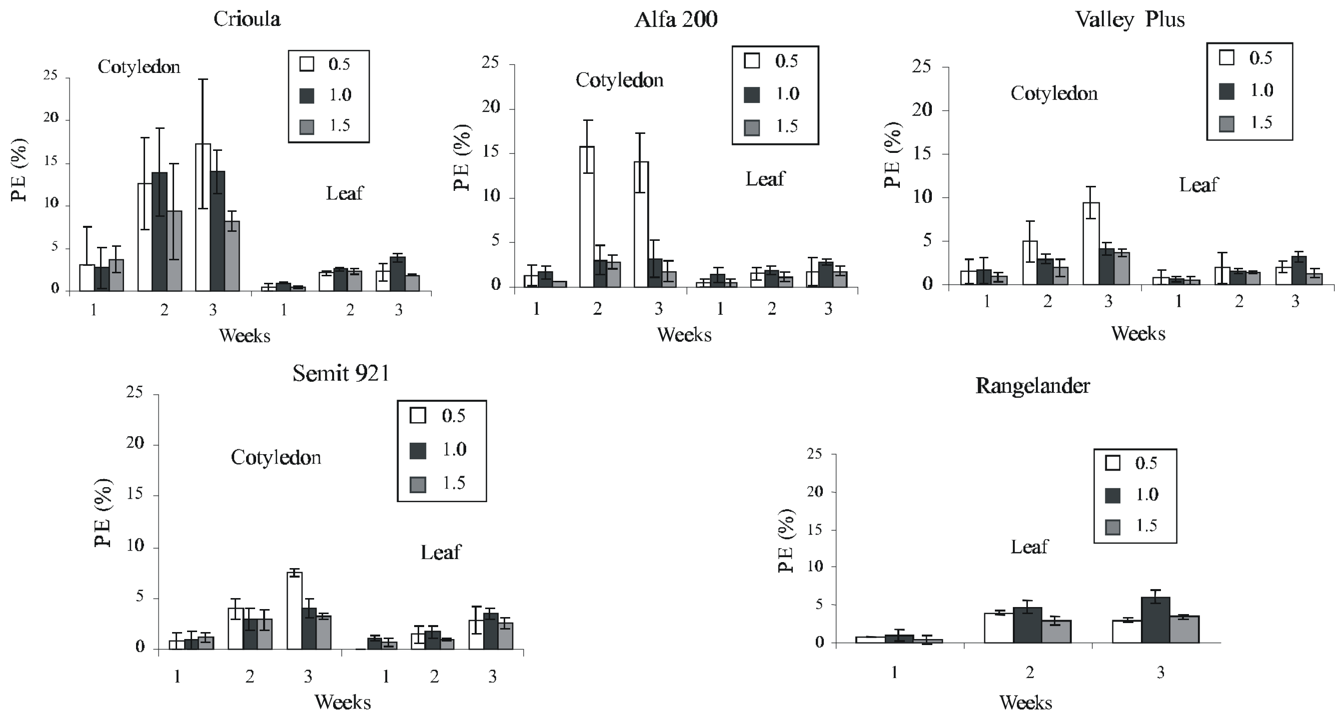

Figure 2 - The influence of the source of protoplasts, plating density (x 10^5) and period of culture on the plating efficiencies (PE) evaluated by the number of microcolonies in relation to total number of protoplasts by microscope field in five alfalfa genotypes.

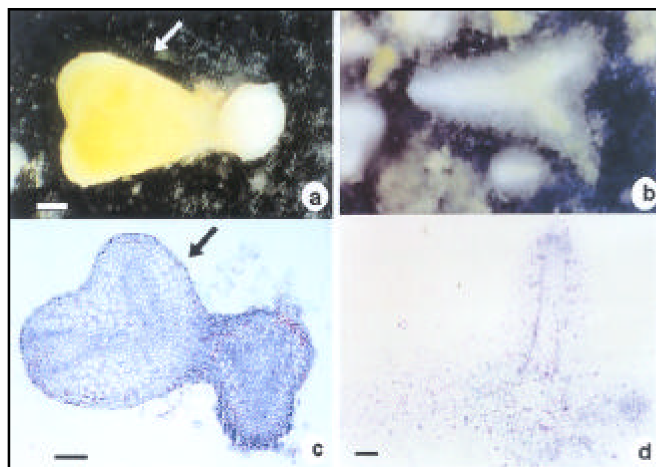

Figure 3 - (**a**) Somatic heart stage embryo (arrow) of *Medicago sativa* 'Rangelander' (bar = 870 µm) and (**c**) its anatomical view (arrow) (bar = 165 µm). (**b**) Callus derived from *Medicago sativa* cultivar Semit 921 protoplasts (93 x) and (**d**) its longitudinal section showing a root primordium (bar = 199 µm).

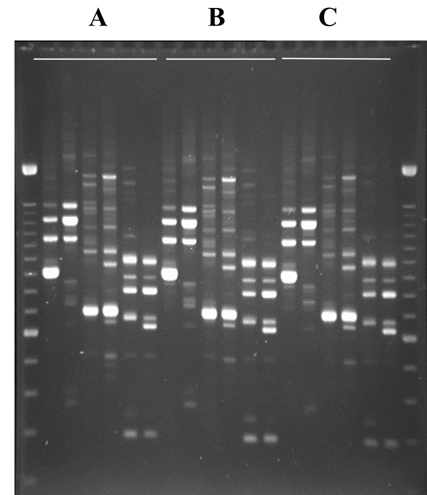

Figure 4 - RAPD profiles of the donor (lanes 2, 4 and 6) and a leaf-protoplast derived plant of 'Rangelander' (lanes 3, 5, 7). Amplifications were performed with primers A01 (lanes 2 and 3), A04 (lanes 4 and 5) and A12 (lanes 6 and 7). Lanes 1 and 20 show the molecular standard (ladder 100 bp). A, B and C correspond to 30, 20 and 10 ng of template DNA, respectively.

Table 2 - Comparison between the protocols used in the present work and that Atanassov & Brown (1984).

	Present work	Atanassov and Brown (1984)	
Explant source	Leaf and cotyledon protoplasts	Leaf and cell suspension protoplasts	Leaf tissues, cotyledons and hypocotyls Cell suspensions
Variety	Crioula, Alfa 200, Valley Plus, Semit 921 and Rangelander	Regen S, Citation and Answer	13 varieties, including Rangelander
Enzymatic digestion	2.0% Cellulase R10, 0.5% Macerozyme R10 in CPW-13 for 17 h	1.0% Driselase, 1.0%, Rhozyme HP 150, 0.1% Cellulase R10 in 72.6 g L^{-1} Manitol for 5-7 h	-
Purification and washing	64 µm followed by 2 low-speed centrifugations in CPW-13	44 µm followed by 2 low-speed centrifugations in EMI with 72.6 g L^{-1} glucose, 200 g L^{-1} $CaCl_2$	-
Plating	Droplets of agarose Bathing medium: K8P 5 d in the dark →25 μEm^{-2} s^{-1} 1.0 x 10^5 (leaf) 0.5 x 10^5 (cotyledon)	Droplets (leaf); Thin layers (cell suspensions) Bathing medium: Kao (1977) with 81 g L^{-1} glucose, 75 mg L^{-1} penicillin G, 5 mg L^{-1} streptomycin 7 μEm^{-2} s^{-1} for 24 h →25 μEm^{-2} s^{-1} 5.0 x 10^3 (leaf) 1.0 x 10^5 (cell suspension)	-
Osmotic	1/3 of the bathing medium was substituted by a mixture of K8P: K8 after 7, 14 and 21 d as follows: 2:1, 1:1 and 0:1	At 17-21 d of culture, 300-500 µL of Kao12 were added i.e. Kao (1977) plus 36 g L^{-1} glucose, 5 g L^{-1} sucrose, 0.2 mg L-1 2,4-D, 1 mg L^{-1} NAA and 0.5 mg L^{-1} BAP	-
Culture	After 28 d, microcalli were transferred to B5h (6 g L^{-1} agar)	After 40-45 d, microcolonies were transferred to Kao_{12}/ B_5h (12 g L^{-1} agar) After 70 d → B_5h Subcultures each 28 d	B_5h
SE induction	SHb plus 11.0 mg L^{-1} 2,4-D, 1.0 mg L^{-1} Kin for 21 d	SHb plus 11.0 mg L^{-1} 2,4-D, 1.0 mg L^{-1} Kin for 21 d	SHb for 21 d
SE growth	Boi2y for 20 d	Subcultures in Boi2y for 21-45 d	Subcultures in Boi2y for 21-45 d
Conversion into plant	½MS (1.5 g L^{-1} Phytagel)	SH or B5 with 10 g L^{-1} sucrose (9 g L^{-1} agar)	SH or B5 with 10 g L^{-1} sucrose (9 g L^{-1} agar)

SE: somatic embryo; Kin: Kinetin

including legume species. Gesteira et al. (2002) used RAPD markers to evaluate genetic stability of regenerants of soybean plants, obtained through somatic embryogenesis using 180 µM 2,4-D. Twenty primers were used to screen 44 regenerants from two soybean cultivars. Three of them were polymorphic for two of the 'Spring'-derived regenerants, with somaclonal variants occurring at a frequency of 4.5%, and four primers were polymorphic for the 'CAC'-1-derived regenerant, with somaclonal variant frequency of 3.57%.

Piccioni et al. (1997) reported the incidence of molecular variation in 9 out of 39 (23%) 'Rangelander' alfalfas derived from somatic embryogenesis. RAPD-based similarity was calculated, and ranged from 72 to 97%. These authors attributed the differences to somaclonal variation. RAPD markers are absolutely useful to detect molecular instability in primary regenerant plants derived via somatic embryogenesis.

This is the first report on plant regeneration from 'Rangelander' alfalfa protoplasts using a protocol defined for alfalfa cell suspension-derived embryogenesis. In summary, the use of agarose-embedded protoplasts at 1 x 10^5 per mL of the culture medium (K8P), and the osmotic reduction protocol led to microcalli development in 28 days. Somatic embryo induction, development and conversion into plant were achieved by using the protocol described by Atanassov & Brown (1984) (Table 2). Because of its agronomical importance and highly morphogenic ability, the use this protoplast-to-plant technology may be useful for genetic manipulation of the cultivar Rangelander.

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