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IN VITRO PERFORMANCE OF MEDICAGO SATIVA L. cv. ZAJEČARSKA 83: DIRECT SOMATIC EMBRYOGENESIS, CELL AND PROTOPLAST CULTURE

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Alfalfa *(Medicago sativa* L.) cv. Zaječarska 83 is a newly registered cultivar in Yugoslavia and its performance in tissue culture was compared to that of cultivars described in other laboratories. The objective of this work was to provide a basis for the use of unconventional methods in further breeding. Somatic embryogenesis was induced in a direct manner, by culture of immature embryos on a medium containing 0.05 mg l^{-1} benzylaminopurine as the sole hormone (Maheswaran and Williams, 1984). Although the genotypic frequency of somatic embryogenesis was rather low (2.74%), the embryogenic lines that were selected produced on the average about 400 somatic embryos per g of tissue, and this capacity has remained constant for more than a year. Procedures for obtaining cell suspensions and protoplasts and for regenerating single cell clones were assayed using callus of hypocotyl origin. It was shown that general techniques (Kao and Michayluk, 1980, 1981) were satisfactory. It is concluded, therefore, that responsive genotypes can be found among the plants belonging to Zaječarska 83 and that the prinicpal *in vitro* techniques could be employed for breeding purposes.

Key words: *Medicago sativa* L., alfalfa, somatic embryogenesis, cell suspension culture, protoplast culture, single cell clones.

Ključne reči: *Medicago sativa* L., lucerka, somatska embriogeneza, kultura ćelija u suspenziji, kultura protoplasta, ćelijski klonovi.

INTRODUCTION

Elaboration of cell and tissue culture methods specifically adapted to species and varieties of economic interest is a prerequisite for their genetic improvement through unconventional methods. Legumes in general were for a long time considered as being recalcitrant to *in vitro* techniques (Thomas and Wernicke, 1978). Since then, however, certain modifications of common procedures resulted in good success with many legumes, alfalfa being a notable example among them (McCoy and Walker, 1984). The most important modifications of the techniques include: (a) The sequential changes of hormone content in the media, for successive phases of callus induction, plant regeneration and embryogenesis (Walker et al., 1979); (b) The elaboration of a medium rich in organic components for cell and protoplast culture (Kao and Michayluk, 1980, 1981); and (c) The choice of the primary explants, mainly leaflets and mesophyll cells, for obtaining protoplasts and for the induction of calli with organogenic capacity (Kao and Michayluk, 1980; Dos Santos et al., 1980; Mezencev, 1982; Lu et al., 1983; Mariotti et al., 1984). Although all important methods have thus been elaborated for alfalfa so far, there exist considerable intra– and intervarietal genotypic differences, which limit their universal application. The genotype remains the major determining factor in the *in vitro* alfalfa performance (Bingham et al., 1985; Reisch and Bingham, 1980; Mezencev and Karelina, 1982; Mitten et al., 1984; Brown and Atanassov, 1985; Meijer and Brown, 1985; Chen et al. 1987; Bianchi et al., 1988).

We present here the results obtained with a recently registered alfalfa variety Zaječarska 83. This variety was produced by a polycross method, using several local populations of the eastern Serbia, which are well adapted to climatic conditions of that region. Zaječarska 83 is claimed to have a high yield, good chemical composition and resistance to many common alfalfa diseases (Mijatović et al., 1983). Plant regeneration in callus cultures and the performance in natural conditions was described in a previous paper (Nikolić et al., 1986). Further progress in obtaining somatic embryogenesis and single cell clones from cell suspensions and protoplasts is described in the present paper.

MATERIAL AND METHODS

Plant material

Seeds of alfalfa, *Medicago sativa* L. cv. Zaječarska 83 were provided by the Institute for Agricultural and Technological Research, Zaječar. The seeds were densely sown in beds of the Beograd Botanical Garden. Immature pods for embryo isolation were collected in the third year from sowing, in September 1987. Cell suspension and protoplasts were obtained from tissues of hypocotyl callus origin, cultured on medium 1 (Nikolić et al.,1986).

Culture media

The culture media for different stages of growth were chosen according to the already published data, among those which were claimed to be suitable for alfalfa cells and tissues. The composition of the media is presented in Tables 1 and 2. All media were autoclaved at 115°C for 20 min.

Tab. 1. – Composition of the culture media

Components	Culture media used for:				
	Callus stock cultures 1	Somatic embryo-genesis 2	Cell sus-pension 3	Proto-plast culture 4	Regene-ration 5
Mineral salts	B	MW	KM	K	SH
Carbohydrates, g l^{-1}					
sucrose	30	40	25	0.125	30
glucose	–	–	5	68.400	–
xylose	–	–	0.250	0.125	–
others[a]	–	–	–	+	–
Agar, g l^{-1}	7.0	7.0	7.0	–	6.0
Agarose, g l^{-1}	–	–	–	4.0	–
Yeast extract, g l^{-1}	–	1.0	–	–	–
Casein hydrolysate, g l^{-1}	–	–	0.50	0.125	–
Proline, mg l^{-1}	–	–	–	–	5750.0
Glycine, mg l^{-1}	2.0	–	–	–	–
Other organics[b]	–	–	+	+	–
Vitamins, mg l^{-1}					
thiamine	0.1	5.0	10.0	10.0	5.0
pyridoxine	0.1	0.5	1.0	1.0	0.5
nicotinic acid	0.5	5.0	1.0	1.0	5.0
m–inositol	–	–	100.0	100.0	1000.0
others[c]	–	–	–	+	–

B = Blaydes (1966); MW = Maheswaran and Williams (1984); KM = Kao and Michayluk (1981); K = Kao (1977); SH = Shenk and Hildebrandt (1972), supplemented with 1.6 g l^{-1} ammonium sulphate; a = 0.125 mg l^{-1} of each fructose, ribose, mannose, sorbitol and mannitol; b = 5 mg l^{-1} Na–pyruvate, 10.0 mg l^{-1} citric acid, 10.0 mg l^{-1} malic acid, 10.0 mg l^{-1} fumaric acid; c = vitamin mixture containing (in mg l^{-1}): 0.5 D–Ca–pantothenate, 0.2 folic acid, 0.01 p–aminobenzoic acid, 0.005 biotin, 0.5 choline chloride, 0.1 riboflavin, 1.0 ascorbic acid, 0.01 vitamin B_{12}, 0.005 vitamin A, and 0.005 vitamin D_3.

Tab. 2. – Hormone content in the culture media

Hormones, mg l^{-1}	Culture media[a]				
	1	2	3	4	5
2,4–D	4.0	–	1.0	0.2	–
NAA	0.5	–	–	1.0	–
BAP	–	0.05	–	–	–
kinetin	3.9	–	–	–	–
zeatin riboside	–	–	0.1	–	–
zeatin	–	–	–	0.5	–

a = see Table 1.
2,4–D = 2,4–dichlorophenoxyacetic acid; NAA = naphtylacetic acid; BAP = 6–benzylaminopurine

Isolation and culture of immature embryos

Immature pods were thoroughly washed with running water, surface sterilized with 70% ethanol and commercial sodium hypochlorite (0.8% active chlorine) for 20 min, and rinsed several times with sterile water. Most embryos were 1–2 mm long in time of excision. They were cultured on medium 2, to which BAP was initially supplemented in two concentrations: 0.05 and 2.0 mg l^{-1}. The explants were cultured in white fluorescent light 7.0 W · m^{-2}, in a day of 16 h, at 25 ± 2°C.

Cell suspension culture

About 2.5 g of callus tissue grown on medium 1 were cut into small pieces two weeks after the transfer to a fresh medium, and transferred to 500 ml Erlenmayer flasks containing 40 ml of liquid medium 3. The flasks were shaken on a horizontal shaker, with 80 rotations per min. After 7 days the suspended tissue was filtered through nylon filters with 200 μm pore size. Cell suspension was maintained by regular weekly transfers to fresh media. The filtered cells were pelleted by centrifugation at 100 x g for 15 min, the pellet resuspended in 2 ml of the liquid medium 3 and plated in Petri dishes ϕ 7 cm, with 10 ml of agar supplemented media, at 38°C. A piece of actively growing callus tissue was put in the middle of Petri dishes, to serve as medium conditioner. The suspension was examined before and after plating, to make sure that it contains only single cells. The Petri dishes were wrapped with parafilm and maintained in darkness, at 25°C.

Protoplast culture

Protoplasts were prepared from callus tissue, two weeks after transfer to a fresh medium. One g of tissue was cut into small pieces and dipped in a Petri dish with 10 ml of enzyme solution, composed of: 1% cellulase Onozuka R10, 0.5% of macerozyme R10 and 0.05% of pectolyase Y23, dissolved in 98 mM mannitol, 400 mM sucrose, 1 mM $CaCl_2 \cdot 2H_2O$ and 4.6 mM MES buffer pH 5.6. The tissue was incubated for 15–16 h, at 25°C in darkness, without shaking. The enzyme–protoplast mixture was consecutively filtered through two nylon gauze filters with 200 and 60 μm pore size. Protoplasts were washed 3 times in a solution containing mineral salts of the medium 4 and 400 mM sucrose. Floating protoplasts were collected after centrifuging once at 100 x g and twice at 50 x g, for 5 min each. Protoplast density was adjusted to 4 x 10^4 per ml, and the suspension was plated with agarose containing medium 4, at 30°C in Petri dishes ϕ 5 cm. The Petri dishes were sealed with parafilm and maintained in darkness at 25°C. After 7 days agarose strips with cell clumps were transferred to a fresh medium. Protocalli which became visible after a month were transferred on medium 5.

RESULTS

Induction of somatic embryogenesis

The capacity of zygotic embryos to give rise to somatic embryo development was assayed using different hormonal content in the initial medium. Altogether 182 zygotic embryos were isolated. Table 3 shows the media in which they were cultured. In all four groups embryo hypocotyls became swollen in a few days and small cell proliferations appeared. After about 15 days green embryo–like structures became visible on proliferated tissue. They occurred in virtually all explants, although the quality of the structures was not the same. Generally the higher BAP concentration (groups 2 and 4)

Tab. 3. – Scheme for the culture of zygotic embyos; medium No. 2 was supplemented with different hormones

Group	No of cultured embryos	Hormones, mg l^{-1}	
		Preculture, 5 days	Culture
1	35	2,4–D 5, kinetin 0.1	BAP 0.05
2	36	2,4–D 5, kinetin 0.1	BAP 2.00
3	52	–	BAP 0.05
4	59	–	BAP 2.00

induced a rather firm callus tissue, from which the embryo–like structures could not be released. The pretreatment with 2,4–D + kinetin (groups 1 and 2) did not produce any improvement in terms of the abundance of organogenesis, while the embryo–like structures tended to look malformed. After a period of 4 weeks all cultures were carefully examined and it became clear that the best results were obtained in embryo group 3. The explants of group 3 produced a soft friable tissue with numerous well discernible organized structures. They were easily released and all stages of embryo development were observed (Fig. 1–3). Most embryos were clearly bipolar, with discernible axis and cotyledons. Out of 52 zygotic embryos of group 3, 5 best lines (2.74%) were selected and maintained in culture. The selection was based on high rate of proliferation, on the numerous somatic embryos produced and on normal appearance of the embryos. Secondary somatic embryos on hypocotyls and roots were frequently noted (Fig. 4). Individual embryos were transferred on hormone–free medium 2, and after 7 days root elongation started (Fig. 5). Plantlets also continued growing in the first medium (Fig. 6) and shoots with trifoliolate leaves developed (Fig. 7). The 5 tissue lines selected have been maintained in culture for over a year. They keep their high embryogenic capacity. A recently examined sample of tissue contained the average number of 430 embryos per g of tissue. Regenerated plantlets could be transferred to a mixture of sand and peat (1:1).

Cell and protoplast culture

Cell suspensions developed in liquid medium after several days of shaking. Cells were rather large, mostly round or oval in shape, with conspicuous nuclei and vacuoles (Fig. 8, 9). The average number of cells was 1.45×10^4 cells per ml in filtered suspensions maintained through several subcultures. Spheric colonies were observed 4 weeks after suspension plating (Fig. 10), and after 2 more weeks they could be transferred to a fresh medium. Several lines of single cell origin were thus established. Green spots were visible upon the transfer on medium 5. However, further growth of buds was poor. The reason may be found in the fact that the callus tissue from which the suspension was obtained was already in the course of losing its organogenic capacity at the time of cell isolation.

The tissue treated with the enzyme mixture produced a suspension of viable protoplasts (Fig. 11, 12). The use of pectolyase Y23 significantly increased the protoplast yield. The average number of protoplasts released was 7.2×10^5 per g of tissue. The cell wall was regenerated 24–48 h after planting. Groups of cells became visible after 7 days. When the protocalli were transferred to the medium 5, green spots indicated bud initiation.

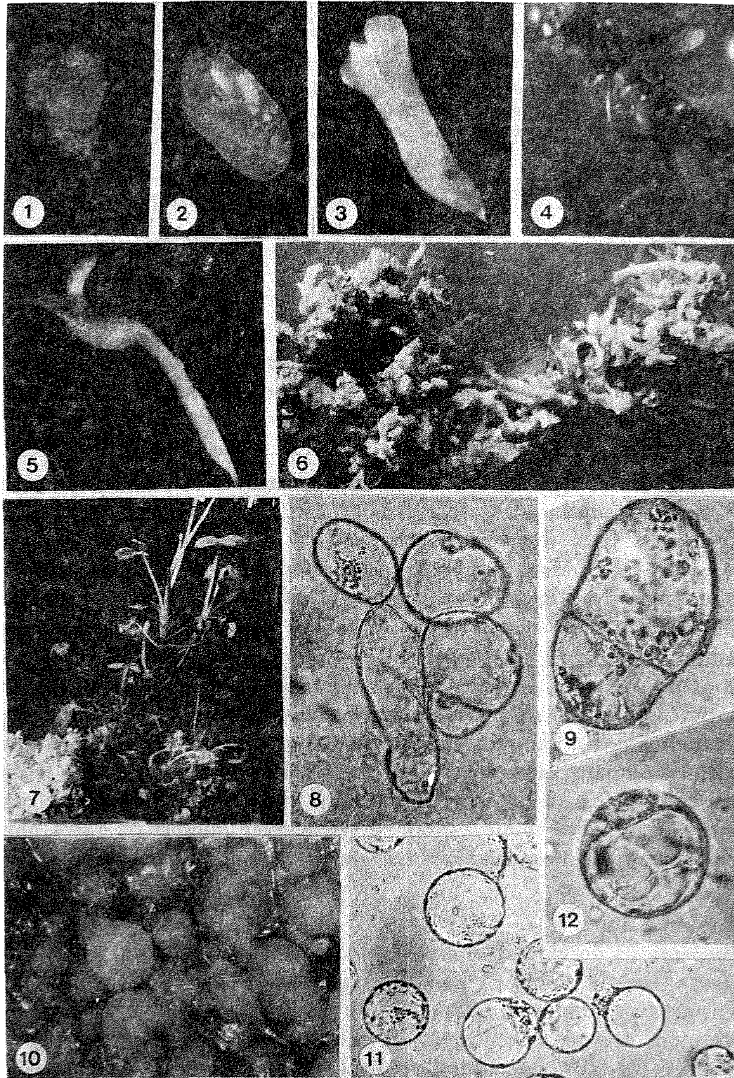

Fig. 1–3: Alfalfa somatic embryos in various stages of development (x 10). Fig. 4: Secondary somatic embryos regenerated on the hypocotyl of an older embryo (x 10). Fig. 5: Plantlet developed from a single embryo transferred to hormone–free medium (x 5). Fig. 6: Embryogenic culture with somatic embryos in various stages of development, 14 months after the isolation of the primary zygotic embryo (x 1.3). Fig. 7: Several embryos elongated into shoots with trifoliolate leaves (x 0.84). Fig. 8: Cell suspension growing in liquid medium (x 128). Fig. 9: Cell division in suspension (x 250). Fig. 10: Single cell clones 4 weeks after plating the cell suspension (x 6.4). Fig. 11 and 12: Alfalfa protoplasts (x 128 and x 250, respectively).

DISCUSSION

The results described in the present paper give evidence on the capacity of a selected alfalfa variety, Zaječarska 83, to respond to some *in vitro* techniques, already elaborated for other alfalfa genotypes. In addition to bud regeneration capacity, which was reported earlier (N i k o l i ć et al., 1986), somatic embryogenesis and the production of single cell clones from isolated cells and protoplasts were proven feasible. Hence it seems that there are no genotypic traits in Zaječarska 83 which would block its responses to commonly used culture conditions.

Somatic embryogenesis in alfalfa has been reported by many authors in the last decade and it seems to be the dominant pattern in plant's regeneration. Apparently four different procedures with slight modifications have been elaborated for achieving somatic embryogenesis, and the preference given to one or the other is likely to be influenced by genotype specificity. Whenever the primary explants were composed of differentiated cells, the sequential changes of growth media in 3–4 steps were required. Protocols for somatic embryogenesis (and organogenesis) (M c C o y and W a l k e r, 1984) involve a rather long phase of (a) callus and (b) embryo (or bud) induction with high hormone concentrations, followed by (c) embryo (shoot) development on a hormone–free, reduced nitrogen–rich medium, and (d) plantlet growth in near–to–normal conditions (W a l k e r et al., 1979; D o s S a n t o s et al., 1980; J o h n s o n et al., 1981; X u et al.,1982; L u p o t t o, 1983; B r o w n and A t a n a s s o v, 1985; N a g a r a j a n et al., 1986; C h e n et al., 1987). M e i j e r and B r o w n (1987) were able to propose a system for somatic embryogenesis working in certain genotypes which takes much shorter and consists only in two steps: embryo induction in primary callus using high hormone concentrations, and embryo differentiation on hormone–free medium. Direct somatic embryogenesis from protoplasts is also possible (K a o and M i c h a y l u k, 1981; L u et al., 1983; D i j a k et al., 1986); this system avoids passage through callus, but nevertheless involves induction with high 2,4–D concentration. In contrast, M a h e s - w a r a n and W i l l i a m s (1984) have elaborated a protocol for direct somatic embryogenesis which is presumably based on the development of predetermined embryogenic cells, present in immature embryos. This procedure avoids both the induction with high hormone concentrations and the passage through callus. Once the somatic embryos are induced, they may be propagated through recurrent embryogenesis, without repeating the induction phase (L u p o t t o, 1986). We have applied similar methods for immature Zaječarska 83 embryos. Unlike to the findings with buckwheat embryos (N e š k o v i ć et al., 1987), we did not observe a higher response in alfalfa after a pretreatment with high 2,4–D concentration. Since only 2.74% of isolated embryos developed embryogenic lines that could be used for propagation purposes, Zaječarska 83 may be ranked among cultivars „possessing regeneration capacity at low genotypic frequency" (M c C o y and W a l k e r, 198). Nevertheless, those 5 selected lines had quantitatively a very good response, which was permanent for more than a year. It is well–known that regeneration from callus induced by high hormone concentrations is frequently associated with cytological aberrations and genetic variability which may be unsuitable for plant propagation (B a y l i s s, 1980). We believe, therefore, that the direct somatic embryogenesis described here is advantageous, since it probably minimizes undesirable effects. Taken together with the possibility of regenerating single cell clones, these techniques provide a promising basis for applying unconventional methods in breeding the cultivar Zaječarska 83.

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Rezime

SLAVICA NINKOVIĆ, JOVANKA MILJUŠ–ĐUKIĆ i MIRJANA NEŠKOVIĆ*

KULTURA MEDICAGO SATIVA SORTE ZAJEČARSKA 83 IN VITRO: DIREKTNA SOMATSKA EMBRIOGENEZA, KULTURA ĆELIJA I PROTOPLASTA

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Lucerka *(Medicago sativa* L.) Zaječarska 83 je nedavno registrovana kao nova sorta u Jugoslaviji i u ovom radu su proučavane reakcije biljaka ove sorte na uslove tkivne i ćelijske kulture *in vitro.* Proučavanja su obavljena sa namerom da se izgradi osnova za primenu nekonvencionalnih metoda u oplemenjivanju lucerke. Somatska embriogeneza je indukovana na direktan način, putem gajenja nezrelih zigotskih embriona na podlozi kojoj je dodat samo jedan hormon, benzilaminopurin (0,05 mg l^{-1}) (Moheswaran i Williams, 1984). Mada je genotipska frekvencija somatske embriogeneze bila dosta niska (2,74%), embriogene linije koje su odabrane obrazovale su u proseku oko 400 somatskih embriona na gram tkiva i zadržale su ovaj kapacitet duže od godinu dana. Za dobijanje ćelijske suspenzije i protoplasta, kao i klonova koji vode poreklo od jedne ćelije, korišćeno je kalusno tkivo izolovano od hipokotila sejanaca. Metodi koji se široko koriste (Kao i Michayluk, 1980, 1981) dali su i ovde zadovoljavajuće rezultate. Na osnovu toga je zaključeno da sorta Zaječarska 83 obuhvata genotipove koji dobro reaguju na uslove *in vitro,* tako da se ove metode mogu primeniti za različite svrhe u daljem radu na oplemenjivanju i selekciji lucerke.