

POLYMORPHISM DETECTION OF *IN VITRO* CULTIVATED *EPILOBIUM* (ONAGRACEAE) SPECIES USING RAPD METHOD

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Abstract: Medicinal plants *Epilobium parviflorum* Schreb. and *Epilobium hirsutum* having a phytopharmaceutical importance, used for the treatment of urinary apparatus and prostate diseases, were alternatively cultivated *ex vitro/in vitro*. By RAPD method, using single primers of arbitrary nucleotide sequence, we attempted to detect genetic polymorphism in purpose of visualizing the putative differences between genetic profiles appeared after *in vitro* cultivation of the subjected species on culture media supplemented with plant growth regulators, in order to select somaclonal variations. The primers we used were able to detect polymorphisms in absence of specific nucleotide sequence information so that polymorphisms function as genetic and molecular markers.

Key words: *in vitro* culture, RAPD, polymorphism, *Epilobium parviflorum*, *Epilobium hirsutum*.

Introduction

Epilobium parviflorum Schreb. (Small Flowered Willow Herb) of Onagraceae family is a hygrophile, perennial species of phytopharmaceutical importance, used for the treatment of urinary apparatus and prostate diseases, less severe scorchs, epidemic irritation or canker. Among the therapeutical effects this species has we can enumerate: inflammation-inhibiting and healing effect on acute and chronic inflammation of the prostate and can help to reduce the gland to its normal size (Steenkamp et al., 2006; Ozasa et al., 2004; Battinelli et al., 2001; Hiermann et al., 1991). Small Flowered Willow Herb contains many compounds that support the prostate: flavorglycosides, especially derivatives of kaempferol, quercetin, and myricetin. *Epilobium parviflorum* also contains Beta-sitosterol, various esters of sitosterol, and sitosterol glucoside have been detected (Balázs et al., 2003). Gallic acid derivatives are also present. Gallic acid and two of the macrocyclic ellagitannins, oenothin A and oenothin B, have been identified as the main constituents responsible for the inhibition of 5-alpha-reductase and aromatase enzymes (Ozasa et al., 2004). These enzymes are considered to play key roles in the cancer of the prostate. Among other actions, it can also be mentioned the uterosedative and bactericide effects (Wichtl and Bisset, 1994), as well as anti-androgenic or antiestrogenic influence.

Epilobium hirsutum (Great hairy willow herb) is a flowering plant belonging to the willowherb genus *Epilobium* (Blamey et al., 2003) in the *Onagraceae* family. The leaves are used to make 'kaporie tea' which is often drunk in Russia. They are edible and they are also sometimes sucked for their salty taste having been used as astringents, but there are some reports of violent poisoning with epileptic-like convulsions as a result of its use. In doses of 1 mg/kg and 3 mg/kg the *Epilobium* extract prolonged the life span of the mice by 156 and 158% accordingly in leucosis P-388 and ascitic tumour of Ehrlich (Voynova et al., 1991). The fresh juice or the powdered root is taken to stop internal hemorrhaging. They stop looseness of the bowel and other fluxes and also nocturnal pollutions.

The main purpose of this study was to analyze the genetic profile of these plantlets cultivated on artificial culture media, using the RAPD amplification method, following the

establishment of an optimal working protocol for *in vitro* micropropagation. We meant to notice some possible genetic modifications that might have appeared due to *in vitro* cultivation.

In purpose of analyzing genetic profiles of vegetal species anonymous low copy number of genomic clones technique was more and more used since 90's (Williams et al., 1990). The polymorphism assays based on the polymerase chain reaction (PCR) require target DNA sequence information for the design of the amplification primers. This technique comes to be expensive considering the time and cost for obtaining this sequence information and therefore it is prohibitive for many large scale genetic mapping applications.

RAPD technique has been considered more practical, requiring the amplification of genomic DNA with single primers of arbitrary nucleotide sequence. As a result of this attempt we obtain the amplification of multiple individualized DNA products. The primers are able to detect polymorphisms in the absence of specific nucleotide sequence information so that polymorphisms function as molecular and genetic markers. In order to reach highly sensitive information on genetic variability profiles by establishing putative differences between genetic patterns and to identify each of the individuals analyzed as a single and specific genetic assembly, this method was continuously improved and adapted to studying of many vegetal species (MacPherson et al., 1993; He et al., 1994). Following this purpose we analyzed several genotypes of *Epilobium parviflorum* and *E. hirsutum* belonging to *Onagraceae* family, having been alternatively grown up *ex vitro* and *in vitro*, trying to observe whether any differences of genetic variability between them were noticed or not. Molecular characterization was accomplished by RAPD markers, where nine to ten base long primers were used for PCR amplification, which was to generate polymorphisms of value for genetic distance analysis. The amplification was accomplished by using 4 primers and the specific working program.

Material and Methods

In vitro culture of *Epilobium parviflorum* Schreb. was induced via seed germination; after being washed in sterile water for 30 min., sterilized with 70% ethilic alcohol for 1 min. and for 15 min. in 10% H₂O₂, seeds were cultivated on ½ MS (Murashige and Skoog, 1962) germination culture media with 0.7% agar. After 15 days caulinar apex, leaf, nodal, internodal, and root explants were excised and transferred on MS medium with 3% sucrose and 0.7% agar. Due to the fact that best results were obtained from nodal explants, we continued using them forward for all the multiplication experiments. In order to obtain callus, 0.5-1.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) was added to culture media. Regeneration and multiplication of shoots was stimulated by using plant growth regulators like naphtylacetic acid (NAA), benzyladenine (BA), kinetin (KIN), N6-(2-isopentyl)adenine (2iP), thidiazuron (TDZ). Culture media composition variants we used (Fig.1): 1.= 0.6 mg/l 2,4-D, 2.= 0.5 mg/l ml 2,4-D + 1.5 mg/l 2iP, 3.= 0.5 mg/l 2,4-D + 1.0 mg/l TDZ, 4.= 0.5 mg/l 2,4-D + 1.5 mg/l BA (All these culture media variants contain 0.2 g/l glutamine and 0.2 g/l caseine), EP13 = control medium with 2 g/l activated charcoal, EP12 = hormoneless control medium, EP7 = 0.2 mg/l NAA + 1.5 mg/l 2iP, EP8 = 0.2 mg/l NAA + 2.0 mg/l KIN 0.2 mg/l NAA + 2.0 mg/l KIN.

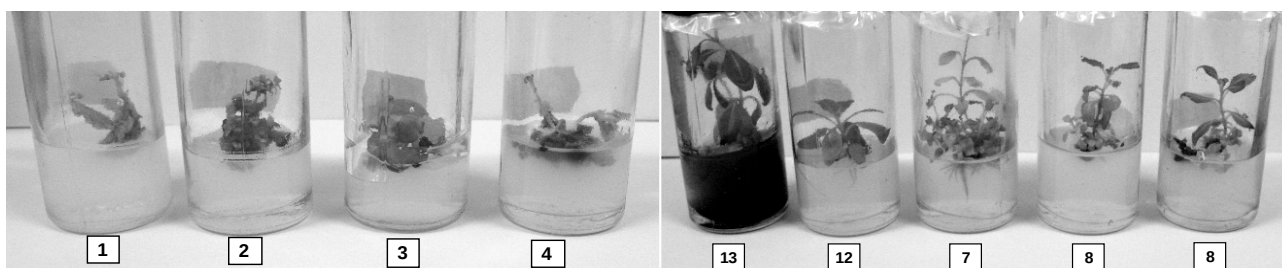


Fig. 1: Culture media variants used for *in vitro* regeneration of *Epilobium parviflorum* Schreb. plants.

For rooting the shoots, MS basic was supplemented with active carbon, without adding growth regulators. pH of culture media was adjusted to 5.7 before autoclavation. Culture jars with plantlets were maintained under 16 hours/day photoperiod conditions, cold light intensity of 40 $\mu\text{mol}/\text{m}^2/\text{s}$ and $25\pm 1^\circ\text{C}$ temperature.

Molecular characterization was accomplished by RAPD markers, where primers of nine to ten bases long were used for PCR amplification, technique that was supposed to generate polymorphism of value for genetic distance analysis, in case that any difference of genetic pattern between *ex vitro* and *in vitro* individuals having been analyzed was noticed. DNA was isolated through the CTAB method (Doyle&Doyle, 1987). Amplification was accomplished by using 4 unspecific primers of different sequences (OPB 04, OPB 07, OPB 17, OPF 04) and the specific working program (Table No.1).

Table 1: Primers characteristics and PCR programs used for RAPD amplification

No.	Name and nucleotide sequence of the primer	No. of Nucl	DNA denaturation Temperature and time	Annealing temp. of the primer (T_m)	Elongation temperature and time	No. of amplif. cycles
1.	OPB-04 GGA CTG GAG T	10	94°C 30 sec.	27.2°C 45 sec.	72°C 1:20 sec.	60
2.	OPB-07 GGT GAC GCA G	10	94°C 30 sec.	32.9°C 45 sec.	72°C 1:20 sec.	60
3.	OPB-17 AGG GAA CGA G	10	94°C 30 sec.	28.2°C 45 sec.	72°C 1:20 sec.	60
4.	OPF-04 GGT GAT CAG	9	94°C 30 sec.	25.5°C 45 sec.	72°C 1:20 sec.	60

Sample composition subjected to amplification was: genomic DNA (50 ng, 5% dilution), (1,5 mM) MgCl_2 , 200mM dNTPs, (200 μM) primer, (1U in 25 μl) Taq polymerase, water, 1X Taq polymerase buffer, 25 μl reaction volume/sample. Control sample did not include DNA.

RAPD amplification products were separated in 1% agarose gel, ethidium bromide stained and visualized by UV. DNA ladder used was 100 bp (up to 1500 kb). Migration condition for electrophoresis 120 V, 1 hour.

Results and Discussion

In vitro culture

Growth and multiplication of explants were comparatively analyzed after 15 and 45 days. At 15 days of micro cultivation, explants grown on MS with 2iP + NAA (var. EP7, Fig. 2A, B) developed normal *E. parviflorum* shaped plantlets while MS medium with BA + NAA (var. EP6) generated extremely vitrified curly shaped plantlets (Fig. 2C, D).

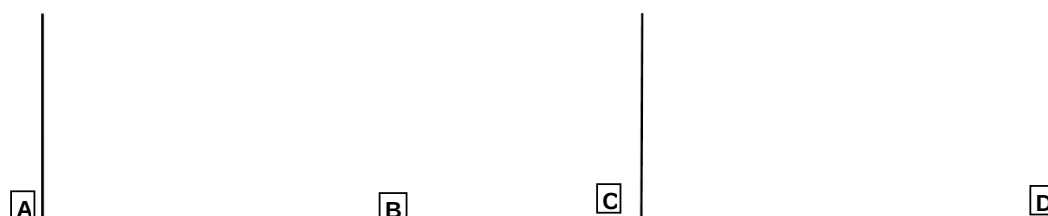


Fig. 2: Comparative analysis of *E. parviflorum* plantlets after 15 days of microcultivation regenerated on MS with 2iP + NAA and on MS with BA + NAA (A, C - Cluster of plantlets, B, D - Separated plantlets).

There was not noticed any relevant difference between the heights that both categories shoots had. Results analysis after 45 days of cultivation *in vitro* revealed a similar situation when *E. parviflorum* plants cultivated on MS including 1.0 mg/l 2iP and 0.15 mg/l NAA developed normal leaves and adventive roots on the stem (Fig. 3A, B). Having a reasonable height, they exhibited a very well rooting process while plants grown on MS with 1.0 mg/l BA + 0.15 mg/l NAA were short, clustered (Fig. 4A, B), they had curly, small leaves, and we noticed an increased vitrification process.

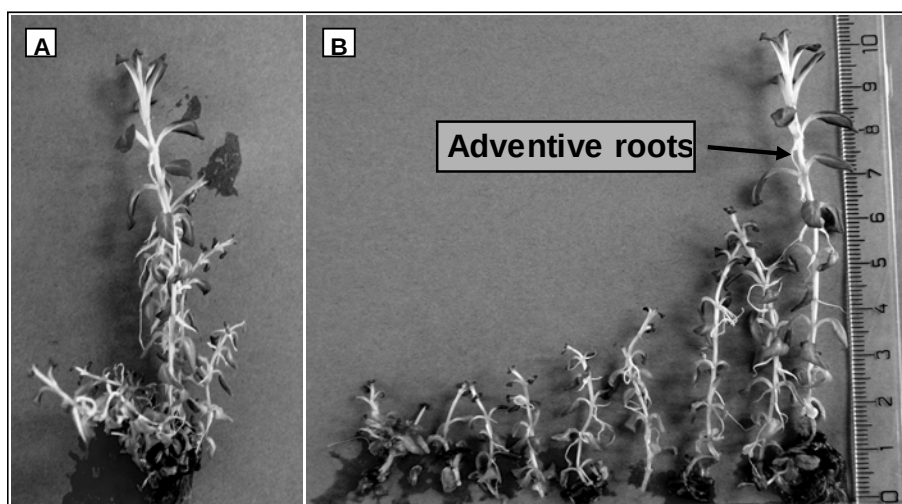


Fig. 3: The effect of MS medium + 2iP + NAA on *E. parviflorum* shoots proliferation after 45 days of culture. (A. Clustered plants. B. Separated plants).

As a conclusion to this study, the effect of two cytokinin/auxin combinations used to influence micropropagation of *Epilobium parviflorum* (A. 1.0 mg/l BA + 0.15 mg/l NAA; B. 1.0 mg/l 2iP + 0.15 mg/l NAA), after 45 days of culture, was displayed as a graphic in Fig. 5. The results obtained showed that BA was the most useful growing factor out of all four hormones used, not only for induction but also for the multiplication of adventive buds and it was followed by 2iP.

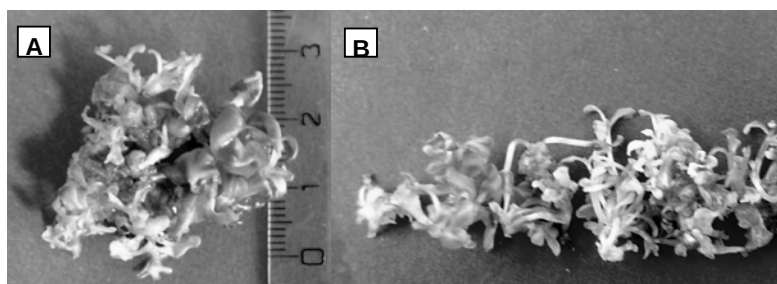


Fig. 4: Proliferation of *E. parviflorum* shoots on MS medium + BA + NAA after 45 days of culture. (A. Cluster of plants. B. Separated plants).

Shoots regenerated on variant EP6 (Fig.5 - F5) are much more shorter than those proliferated on MS with 2iP included, represented in var. B. (var. EP7), where we notice that the number of micropropagated shoots and their length are in a reverse proportion compared to the direct proportion of var. EP6. After 45 days of culture, one shoot on EP6 medium variant multiplies 20 shoots, most of them rootless and being about 2.0 mm length (Fig. 5 and Fig. 6) while var. EP7 generated a number of 5.8 shoots/explant, about 7.0 cm length and it is also evident an increased rooting process (3-4 roots/explant).

In order to increase quality of shoot regeneration and rooting there were used several combinations of culture media: hormone free MS, MS + NAA, hormone free MS + activated charcoal (AC), having several concentrations each of them. Only two variants corresponded to our intentions (EP10 – MS, EP13 – MS + 2.5 g/l AC), assuring an acceptable regeneration of shoots and stimulating formation of a reliable root system (Fig.5 - F6), both aspects being essential for accommodation of plants to *ex vitro* conditions. Positive effect of AC on shoot rooting was demonstrated several years ago (Hussey, 1986). Our conclusions lead to the idea that increasing AC concentration damages shoot regeneration by reducing surface of shoot leaves.



Fig. 5: F5 - Micropropagation of *E. parviflorum* after 45 days on two var. of culture media (cytokinin/auxin combinations): A- 1.0 mg/l BA + 0.15 mg/l NAA; B- 1.0 mg/l 2iP + 0.15 mg/l NAA (left image). F6 - *In vitro* regeneration and rooting of *E. parviflorum* (after 45 days): 1- on MS hormone free medium, 2- on MS + 2,5 g/l active carbon.

RAPD molecular analysis

RAPD amplification products of *Epilobium parviflorum* plants multiplied *in vitro* and transferred *ex vitro* indicated a lack of polymorphism in most of the cases (Fig. 6 A., B., Fig. 7 A. – positions 2, 3, 4). Monomorphic amplification suggested an increased genetic homogeneity of regenerated plants related to donor plant. Disposition of samples was identical on each of the four images of agarose gel presented (Fig. 6, 7) as following: pos.1-100 bp DNA ladder; 2, 3, 4 *in vitro E. parviflorum* (cultivated on medium 6 - 2,4-D, medium 7 - 2iP and medium 13 - hormone free, as presented in material and method section); 5, *ex vitro E. parviflorum*; 6, 7, 8 *ex vitro E. hirsutum*; 9, 10, 11 *ex vitro* acclimatized (from *in vitro*) *E. parviflorum*; 12, negative control.

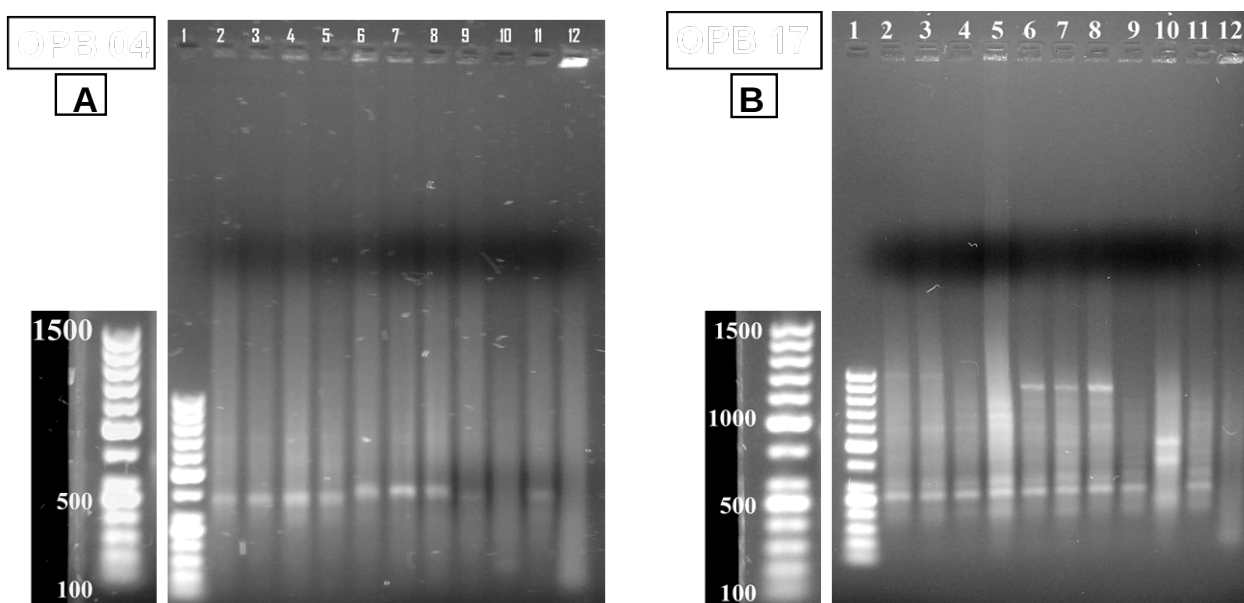


Fig. 6: (A.) RAPD products resulted by amplification with primer OPB 04: pos.1-100 bp DNA ladder; 2, 3, 4 *in vitro E. parviflorum* (culture media 6, 7, 13); 5, *ex vitro E. parviflorum*; 6, 7, 8 *ex vitro E. hirsutum*; 9, 10, 11 *ex vitro* acclimatized *E. parviflorum*; 12, control. (B.): RAPD products obtained with primer OPB 17. Samples have the same disposition like in Fig. 6A.

For some of the *ex vitro* acclimatized *E. parviflorum* plants (Fig. 6B. positions 9, 10, 11) showing a slight polymorphism (sample on position 10 gave three amplification bands of 500 bp, 800 bp and 1000 bp, different from samples on positions 9 and 11) genetic profile differences resulted from the fact that *ex vitro* acclimatized (from *in vitro*); plants having been analyzed derived from different donor plants, which implies a DNA variability itself, indicating a natural genetic variability.

Samples amplified with OPF 04 primer (Fig. 7A.) showed a monomorphic and homogenous display for all *in/ex vitro* plants, the amplification band in each of the cases being situated around a value of 900 bp. A single exception recorded on position 10 (*ex vitro* transferred *E. parviflorum*), where the amplification product was about 600 bp. (the explanation for this result was detailed previously).

Amplifications with OPB 07 distinguished a low polymorphism (Fig. 7B.) *in vitro* between individuals cultivated on M6, M7 and M13 culture media variants. In addition to other commentaries made we can mention that it could exist a low influence of growing factors 2,4-D and 2iP on genetic profiles (positions 2, 3) of *in vitro* cultivated plants.

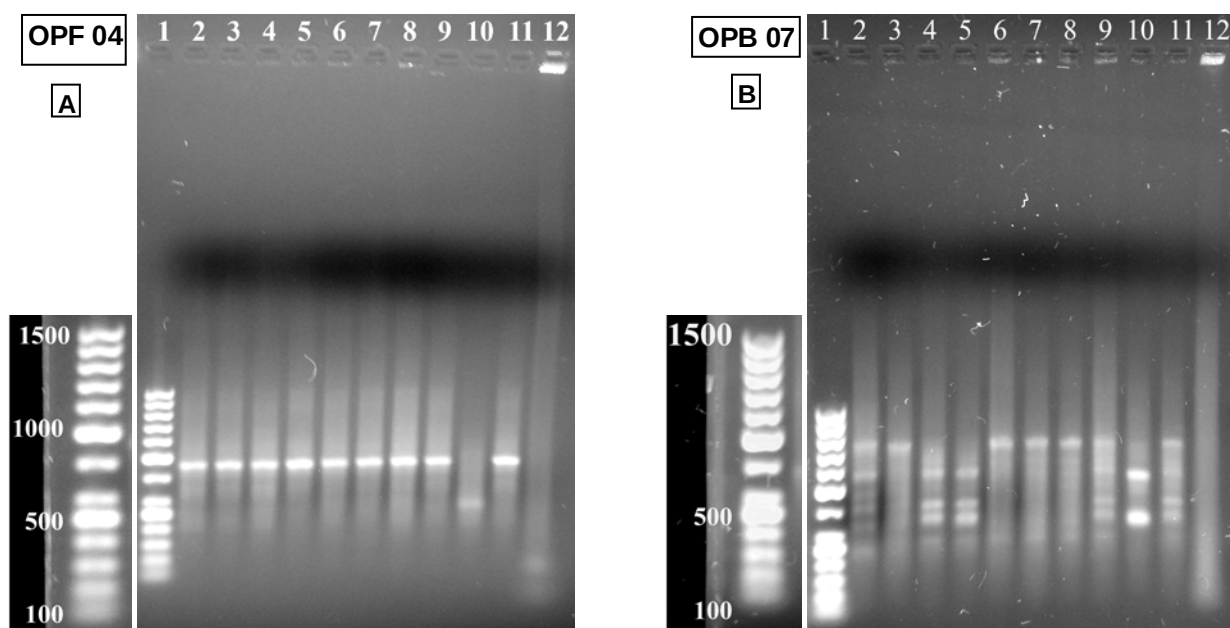


Fig. 7: (A.) RAPD products obtained by using primer OPF 04: pos.1-100 bp DNA ladder; 2, 3, 4 *in vitro* *E. parviflorum* (culture media 6, 7, 13); 5, *ex vitro* *E. parviflorum*; 6, 7, 8 *ex vitro* *E. hirsutum*; 9, 10, 11 *ex vitro* transferred *E. parviflorum*; 12, control. **(B.): RAPD products obtained by amplification with primer OPB 07.** All these samples have the same succession like in Fig. 7A.

Plants regenerated on culture media without growing factors show the same profile as those acclimatized *ex vitro*, which is normal (positions 4 and 5). There are also some bibliographical sources mentioning the fact that slow hypothetical polymorphisms can happen due to some primer misspair (with one position more or less), which is caused by a punctual mutation, an insertion or a deletion of a short sequence, or a transposable element (Peschke et al., 1991). It can be also noticed a low polymorphism of *E. parviflorum* plants transferred *ex vitro* from M6, M7 and M13 culture media (Fig. 7B. pos. 9, 10, 11), related to the fact that they are originated from different donor plants (also see Table No. 2 for amplification results).

Table 2: Number of amplification bands resulted using each of the four primers.

Primers used for amplification	In vitro <i>Epilobium parviflorum</i> M6, M7, M13 (somaclones)	Ex vitro <i>E. parviflorum</i> (individual)	Ex vitro <i>Epilobium hirsutum</i> (individuals)	Ex vitro transferred <i>E. parviflorum</i> (originated from different donor plants)
OPB 04	1 band	1 band	1 band	1 band
OPB 17	1 band	1 band	2 bands	1-3 bands
OPF 04	1 band	1 band	1 band	1 band
OPB 07	1-3 bands	3 bands	1 band	2-4 bands

Conclusion

The results obtained showed that BA growing factor was very useful for initiation and multiplication of adventive buds, but it regenerated short, curly, vitrified plants. In contrast, 2iP was more proper to well development of plants. Activated charcoal added to culture media assured a better regeneration of shoots and stimulated the root system formation.

Following RAPD polymorphism analysis using four primers (OPB 04, OPB 07, OPB 17, OPF 04) it can be concluded that plants regenerated *in vitro* on different culture media compositions showed no polymorphism, like we expected; little supposed polymorphic distributions can be ascribed either to the influence that growing factors had on their regeneration or to their provenience from different donor plants, indicating a natural genetic variability, doubled by some putative miss pairing of the unspecific primers used.

As a future purpose, this work could be extended by analyzing with the same primers some new *in vitro* *E. hirsutum* individuals as well, to be cultivated on the same culture media we used for *E. parviflorum*, for a more appropriate comparative study of polymorphic distribution.

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STUDIUL POLIMORFISMULUI GENETIC PRIN METODA AMPLIFICĂRILOR RAPD LA SPECII DE *EPILOBIUM* (ONAGRACEAE) CULTIVATE *IN VITRO*

(Rezumat)

Hărțile genetice bazate pe utilizarea unor markeri moleculari ADN, care cuprind fragmente amplificate între care există o diferență mică de greutate moleculară, sunt frecvent utilizate în analiza profilului genetic al unor specii vegetale. Pentru vizualizarea polimorfismului unui număr mic de copii anonime din genomul speciilor analizate se folosesc clone genetice, provenite din aceiași indivizi, care sunt comparate între ele. Analiza polimorfismului genetic, bazată pe principiul reacției polimerazice în lanț (PCR), necesită informații despre secvențe țintă de ADN, pe baza cărora sunt construite amorsele folosite în amplificare. Metoda RAPD este mai practică și se bazează pe amplificarea unor secvențe de ADN genomic cu amorse unice care au o secvență nucleotidică arbitrară. Acestea sunt capabile să pună în evidență polimorfismul în absența unor secvențe specifice de nucleotide și funcționează ca niște markeri moleculari și genetici. Speciile cu aplicații medicinale *Epilobium parviflorum* Schreb. și *Epilobium hirsutum*, care au o importanță fitofarmaceutică și sunt folosite pentru tratarea disfuncțiilor aparatului urinar și prostatei, au fost cultivate alternativ *ex vitro* și *in vitro* și analizate din punctul de vedere al polimorfismului genetic. Am încercat să punem astfel în evidență eventualele modificări ale profilului genetic al acestor specii, survenite în urma cultivării acestora pe medii de cultură artificiale suplimentate cu regulatori de creștere vegetali.