

IN VITRO CONSERVATION OF THE CRITICALLY ENDANGERED TAXON *Convolvulus persicus* L. AND REGENERANTS EVALUATION

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Abstract. We had the aim to test *in vitro* reactivity of *Convolvulus persicus* for conservative purpose. Fragments of roots, hypocotyls, stem and leaves were used as explants. Modified Chu formula (1978) was used as a base medium owing to the lower content of salts comparing to MS formula. Two main directions of *in vitro* development were taken into consideration in our experiment: callus induction on auxins added media and shoots development on media supplemented with higher level of cytokinins and lower level of auxins. *In vitro* developmental processes varied from the induction of a non-regenerative callus or a friable proembryogenic callus, to indirect and direct morphogenesis process, depending on the type of explants and medium variant used. The best regeneration rates were registered in the case of medium Chu supplemented with kinetin (kin) and indole acetic acid (IAA) and active charcoal and medium Chu added with benzyl-amino-purine (BAP), dichlorophenoxyacetic acid (2,4-D) and active charcoal. Several clones were regenerated having the origin in different seeds. The uniformity of the individuals belonging to two different regenerated clones was evaluated using isoenzymes markers as peroxidase, malate-dehydrogenase and isoesterase. Among the analysed biochemical markers, peroxidase showed a relevant spectrum of bands, the regenerants being identical concerning this marker.

Keywords: *Convolvulus persicus*, *in vitro* conservation, morphogenesis, isoenzymes spectrum.

Rezumat. Conservarea *in vitro* a taxonului critic periclitat *Convolvulus persicus* L. și evaluarea regeneranților. A fost studiată reactivitatea *in vitro* a speciei *Convolvulus persicus* în vederea conservării. În acest scop s-au utilizat diferite tipuri de explante: rădăcini, hipocotil, fragmente de tulpină și frunze. Explantele s-au cultivat pe mediul Chu (1978) care prezintă o concentrație de săruri scăzută în comparație cu mediul Murashige-Skoog. În cadrul experimentelor s-au urmărit două direcții de dezvoltare *in vitro*: inducerea calusării pe medii adiționate cu auxină și regenerarea lăstarilor pe medii suplimentate cu concentrații ridicate de citokinine și concentrații scăzute de auxine. Procesele de dezvoltare au variat în funcție de tipul de explant și varianta de mediu utilizată de la calus neregenerativ, la calus friabil proembriogen, la morfogeneza directă și indirectă. Cele mai bune rate de regenerare au fost înregistrate în cazul variantei constând în mediul Chu adiționat cu kinetină, acid indolil acetic (IAA) și cărbune activ și a celei bazate pe aceeași formulă și suplimentată cu benzil amino purina (BAP), acid diclorofenoxiacetic (2,4 D) și cărbune activ. Mai multe clone au fost regenerare cu originea din semințe diferite. Uniformitatea indivizilor aparținând a două clone diferite a fost evaluată folosind ca markeri enzimatici peroxidazele, malat dehidrogenazele și izoesterazele. Între markerii biochimici analizați, peroxidazele au evidențiat un spectru de benzi relevante, regeneranții fiind identici.

Cuvinte cheie: *Convolvulus persicus*, conservare *in vitro*, morfogeneza, spectrul izoenzimatic.

INTRODUCTION

Conservation of biodiversity is important for the future of humanity and the Planet Earth, so it “is integral to the biological inheritance of many people and the critical components of healthy ecosystems that are used to support economic and social developments (KASSO & BALAKRISHNAN, 2013).

Global diversity is affected by numerous, varied and interacting factors, mainly as consequence of human activities as habitats loss, fragmentation, degradation, harvesting and exploitation (YOUNG et al., 2007) and also due to natural factors as climatic changes, invasive species, and pests.

Biological factors as problems with sexual reproduction and genetic drift conducted to small, fragmented and isolated remnant populations (LACY et al., 2000).

Owing to these anthropogenic or natural factors, many plant species became threatened, and for this reason, different measures at international and national levels were imposed.

Two main directions of conservation, which are complementary, were developed: *in situ* and *ex situ* strategies; the last one was outlined in the Article 9 of the Convention on Biological Diversity (GLOWKA et al., 1994) and in Target 8 of the Global Strategy of Plant Conservation (2012),

Owing to the heavy loss or decline of species, populations and ecosystem composition, leading to biodiversity reduction due to habitat destruction, *in situ* methods alone are insufficient for saving endangered species needing complementary approaches (CRUZ-CRUZ et al., 2013).

Progresses made in plant biotechnology can provide tools for collection, multiplication and conservation of plant species during different time intervals using *in vitro* cultures, which complement the other *ex situ* conservation methods (field collections, botanical gardens, seeds banks).

In vitro collecting methods are less invasive because they do not affect the viability of the whole plant, relying on the fragments detached from vegetative or reproductive organs or seeds, allowing an efficient sampling (PENCE, 2005).

In the case of reduced population or when seeds are not available or plant has low seeds viability, the vegetative multiplication and even variability, if it is necessary, can be ensured by *in vitro* methods, which are convenient for *ex situ* conservation purpose (BENSON, 1999; REED et al., 2011).

Plant biotechnology have already proved an important role for *ex situ* preservation of plant diversity around the world (TANDON & KUMARIA, 2005; HOLOBIUC, 2005; SARASAN et al., 2006; BUNN et al. 2007; PILLATTI et al., 2011; ENGELMANN, 2011; REED et al., 2011; ASHMORE et al., 2011; GONZÁLEZ-BENITO & MARTÍN, 2011; KASSO & BALAKRISHNAN, 2013).

As *ex situ* conservation strategy, *in vitro* methods can provide the conservation on short-term based on tissue culture induction, medium-term preservation relied on slow-growth procedures (HOLOBIUC & BLINDU, 2006; HOLOBIUC et al., 2009; 2010) or long-term using cryopreservation techniques (REED et al., 2000; SARASAN et al., 2006; CRUZ-CRUZ et al., 2013), the initiation of *in vitro* cultures and multiplication being the first step in this *ex situ* approach.

Convolvulus persicus L. taxon has the conservation status as critically endangered for Romanian Flora (DIHORU & NEGREAN, 2009).

The general distribution of *C. persicus* is Asia and Asia Minor, Caucasus and the Black Sea coast in Romania and Bulgaria (FĂGĂRAS, 2012; TZONEV et al., 2005). This taxon is a Ponto-Caspian element, growing in Romania on sandy areas from Mamaia, Agigea, Eforie, Movila (CT), Sf. Gheorghe, Mila 15, Rosetti, Letea, Caraorman, Sulina, Cardon, Rosetti, Portita, Perisor (TL).

This species has generally a limited distribution and small size of the populations; tourism can affect the populations in the coastal areas.

This species may have a decorative value and was used in traditional medicine for insomnia and respiratory diseases treatment, being surnamed 'fisherman tea', containing calming and emollient principles.

C. persicus is a perennial plant with a short woody rhizome, with stems of 15–40 cm high, with elliptic-ovoid leaves and white pubescent flowers consisting in white corolla, with 5 pubescent stripes on the outer side. Fruit are ovoid with 1–2 black seeds.

Conservation measures which already were taken: the species is protected and included in the Critical List of Vascular Plants from Romania (OPREA, 2005), in the Red Book of Vascular Plants from Romania (DIHORU & NEGREAN, 2009), and in Bulgaria in the Red List of Bulgarian Vascular Plants (2009).

Conservation measures recommended for this taxon are: elaboration of an action plan for *in situ* conservation; the *ex situ* preservation, the multiplication and reintroduction in the natural habitats from Romania and Bulgaria could also significantly help the conservation of this species.

Our aim was to introduce *in vitro* for conservative purpose, to micropropagate and to evaluate the regenerants using biochemical markers as isoenzymes electrophoretic spectrum.

MATERIAL AND METHODS

Seeds collected from plants growing in Sulina were pre-sterilized in 70⁰ ethylic alcohol for 1 minute and then in 0.1% HgCl₂ for 15 min, followed by three washing in sterile distilled water.

Sterilized seeds were cultured on 1/2 MS medium (MURASHIGE & SKOOG, 1962) and germinated in 3 weeks after incubation at 16/8 photoperiod of illumination and 25⁰C temperature regime.

After 2 weeks, from germinated seedlings, different organs (roots, hypocotyls, stem and leaf fragments) were used as explants and cultured on different media variants based on modified Chu formula (CHU, 1978) added with B5 Gamborg vitamins (GAMBORG, 1968), 30 g/l sucrose and active charcoal at 0.5 g/l (Table 1).

The culture media were adjusted at pH 5.9 and sterilized at autoclave at 121⁰C and 1.2 atm.

Because this species usually grows on sandy and salty substrate, we tested the addition of NaCl in one medium variant to check if its presence had a beneficial *in vitro* effect;

Five explants/ 6 cm Petri dish in 3 repetitions were cultured for every variant. The fragments of roots, hypocotyls, petioles and leaves were placed horizontally, while stem fragments were cultured vertically. The subcultures were made after 2 months. The regenerated shoots were cultured in glass jars of 7 cm diameter and 10 cm height.

The cultures were maintained at 2,000 lux illumination and 16/8 photoperiod and 25⁰C temperature regime using cool white fluorescent lamps.

In vitro response was scored after 60 days of culture concerning the mean number of regenerants/explants in ten media variants. Data were statistically analysed using Daniel's XL Toolbox version 6.52 program (<http://xltoolbox.sourceforge.net>) and for comparison of variants Posthoc test Bonferroni-Holm was applied.

Shoots were grown on MS medium without growth factors and supplemented with 20 g/l sucrose and also on M11 variant based on modified Chu formula added with active charcoal, 20 g/l sucrose and low level of auxin NAA.

Several clones were regenerated having the origin in different seeds.

The stability of individuals belonging to two different clones (A and B) regenerated on variant M10 was analysed concerning isoenzymes markers using entire shoots for processing the protein extracts.

Preparation of total protein extract and isozymes electrophoresis

The enzymes extraction was carried out in 50mM phosphate buffer, pH=7, EDTA 2mM, 4% PVP. The tissues samples were ground with quartz sand and the homogenate was centrifuged at 15,000 rpm, for 20 min; the supernatant was used for electrophoresis to detect several enzymes: peroxidases (POX), esterases (EST), catalases (CAT), alkaline phosphatases (AKP) and malate dehydrogenase (MDH).

The polyacrylamide gel electrophoresis was prepared using a 10% (7% in case of catalases) running gel and the running buffer was 0.025M Tris-Gly, pH 8.3.

Gel electrophoresis staining bands

For *peroxidase* detection, it was used H₂O₂ as substrate in acetate buffer and benzidine (WANG & WANG, 1989).

In the case of *catalases*, we used as substrate 0.003% H₂O₂ prepared in 0.01 M phosphate buffer, at pH=7, added with K₃(Fe(CN)₆) and FeCl₃ (IORDACHESCU & DUMITRU, 1988).

For *esterases* detection, it was used as substrate a solution of α naphthyl acetate and Fast Blue RR prepared in 0.1 M phosphate buffer, at pH=6.5 (BACH, 1989 modified.) The bands are stained in red-violet.

For *alkaline phosphatases* assay, the substrate was Na α naphthyl phosphate prepared in Tris –citrate, at pH=8.3. The bands are stained in brown.

For *malate dehydrogenase* (MDH), the reaction mixture consisted from 2M malic acid in Tris-HCl buffer, pH=8, 2mM EDTA, 0.5M MgCl₂, 1% NAD, than 1% NBT, 1% PMS, 1% MTT. The bands are coloured in violet.

Table 1. Media tested for *in vitro* cultures of *Convolvulus persicus*.

Components		Variants										
		1	2	3	4	5	6	7	8	9	10	11
Macroelements		Chu	Chu	Chu	Chu	Chu	Chu	Chu	Chu	Chu	Chu	Chu
Microelements		Chu	Chu	Chu	Chu	Chu	Chu	Chu	Chu	Chu	Chu	Chu
B Complex Vitamins		B 5	B5	B5	B5	B5	B5	B5	B5	B 5	B 5	-
Sucrose (g/l)		30	30	30	30	30	30	30	30	30	30	20
Growth factors (μM/l) (μM/l)	BAP	-	-	-	-	4.4	4.4	22	-	-	-	-
	Kin	-	-	-	0.84	-	-	-	-	-	9.2	-
	2-IP	-	-	-	-	-	-	-	-	9.8	-	-
	Ads	-	-	-	-	-	207.2	-	207.2	-	-	-
	NAA	-	-	-	-	0.54	0.54	-	0.54	1.08	-	0.054
	IAA	-	-	-	-	-	-	-	-	-	1.14	-
	IBA	-	-	-	2	-	-	-	-	-	-	-
	24-D	9.04	-	-	4.52	-	-	9.04	-	-	-	-
2,4,5-T		-	7.8	7.8	-	-	-	-	-	-	-	
Other compounds (g/l) compounds (g/l)	NaCl	-	-	2.5	-	-	-	-	-	-	-	
	AC	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	

Legend: BAP-benzyl amino purine, Kin-kinetin, 2-IP- isopentenyl adenine, Ads, adenine sulphate, NAA, α -naphthyl acetic acid, IAA- β indole acetic acid; IBA- indole butyric acid, 2,4-D-acid 2,4- dichlorophenoxyacetic acid, 2,4,5-T- 2,4,5-trichlorophenoxyacetic acid, NaCl- sodium chloride, AC- active charcoal.

RESULTS AND DISCUSSIONS

Referring to *Convolvulus persicus*, no works concerning *in vitro* culture for conservative purpose have been reported so far.

To test *in vitro* reactivity, we used several types of explants as roots, hypocotyls, stem and leaf fragments.

Chu formula was chosen as a base medium owing to the lower content of salts comparing to MS formula.

Two main directions of *in vitro* development were taken into consideration in our experiment: callus induction on auxins added media and shoots development on media supplemented with higher level of cytokinins and lower level of auxins.

In vitro developmental processes varied from the induction of non-regenerative callus or a friable proembryogenic callus, to indirect and direct morphogenesis, depending on the type of explants and medium used (Table 2).

In the case of auxin-supplemented media (M1-M4), only roots underwent morphogenesis (Fig. 3), while leaf and stem fragments generated only callus (Fig. 2). Root fragments cultured on auxin –added media M1-M3, generated shoots (2-3/ explant), but hypocotyl fragments generated only friable green callus.

The regeneration frequency (the number of regenerative explants/total of inoculated explant x100) was lower, about 35% for M4 variant in the case of roots, in the other variants as M1-M3 using roots as explants the rate of regeneration varied between 50 and 70 % and in M5-M10 using stem fragments, the regenerative rate was about 90-100 %.

The culture of leaf and petiole fragments on M1-M4 variants conducted only to callus development. The same explants generated proembryogenic callus on M1 variant, which did not develop further, and also a friable green –white callus on M2 and M4 variants. This friable callus can be further evaluated and tested for secondary metabolites production. Using stem fragments, also a green compact non-regenerative callus was induced at the end of the explants on media M1-M4. Concerning regenerative response, the shoots formation was evaluated on M1-M10 variants (Fig. 1) using roots fragments as initial explants cultured on M1-M4 and stem fragments cultured on M5-M10.

The root and stem fragments had a positive *in vitro* response, while leaves and petioles were less reactive and not appropriate as a source for micropropagation of plants.

The culture in the presence of different combinations of growth factors with the cytokinin dominance (M5-M10) conducted to indirect (Fig. 4) or direct morphogenesis (Figs. 3, 5) with a mean number of shoots/explants varying between 2 and 4 (Fig. 1). This developmental way occurred in *C. persicus* slower, the number of regenerants per initial explant being scored just after 2 months.

The presence of NaCl at 0.25 % in the culture medium M3 did not influence the *in vitro* response.

The culture on M7 variant determined the formation of callus at the end of the stem fragments, which also generated shoots from lateral meristems. Their culture on regeneration media M5-M10 conducted to morphogenesis process; the best regeneration (significantly different) were registered in the case of M10 variant supplemented with kinetin and IAA (Fig. 5), and M7 added with BAP and 2.4-D.

An improved regeneration rate in *C. persicus* was obtained on M10 variant, with a maximum of 5 shoots/explant registered after two months.

The regenerants were further cultured both on MS medium, added with 20 g/l sucrose and also on MS medium based on Chu formula, added with AC and NAA, the last one which stimulated both rooting process (long and well-developed roots) and lateral shoots formation on already regenerated shoots. The frequency of shoots rooting was 100% on M11 variant, while on MS medium the frequency was lower – about 30% and number reduced at 1-2 poorly developed roots.

In the related species *Convolvulus scindicus*, ABBAS et al., (2012), it was evaluated the *in vitro* response also using germinated seeds as initial plant material. Nodal segments were used as explants and cultured on MS media added with various combination of BAP and NAA and BAP, kin and NAA.

The regeneration frequency was quite limited and the maximum number of shoots /explant was close to our record – 4.7. Further, using a combination of BAP (2.5 mg/l), kin (0.5 mg/l) and NAA (0.5 mg/l), they succeeded to regenerate a mean of 8.2 shoots/explants. The rooting occurs with the best frequency (67%) at 2 mg/l IAA supplemented medium, but with weak root formation.

Shoots culture on variant M11 consisting in modified Chu medium supplemented with 20 g/l sucrose, active charcoal 0.5 mg/l and low level of NAA conducted to improved rooting rate(100%), well developed roots and stimulation of lateral branching, which can ensure material for secondary multiplication (Fig. 6).

In *C. persicus*, regenerated shoots grew well on MS added with 20 g/l sucrose without growth factors, but rooted poorly on this variant (Fig. 7).

Table 2. *In vitro* behaviour of *C. persicus* using different explants after 60 days of culture.

Variants	Roots fragments and hypocotyls	Stem fragments	Fragments of petioles and leaves
1	direct morphogenesis from roots and callus from hypocotyls	green callus at the ends of explant	green yellow friable proembryogenic callus
2	direct morphogenesis from roots and callus from hypocotyls	green callus at the ends of explant	green non-regenerative callus
3	direct morphogenesis from roots and callus from hypocotyls	callus at the end of explants	green non-regenerative callus
4	poor shooting and callus formation	non-regenerative green callus	green non-regenerative callus
5	-	buds development	green callus, indirect morphogenesis
6	-	indirect morphogenesis	green callus without regeneration
7	-	direct morphogenesis	yellow friable callus
8	-	direct morphogenesis	Indirect morphogenesis wit lower rate
9	-	indirect morphogenesis with friable callus, without root formation	indirect morphogenesis
10	-	direct morphogenesis	indirect low morphogenesis

The newly formed lateral shoots can be also used as source of explants for further culture cycles contributing to improve the regeneration rate.

Concerning rooting process, RATHORE et al., 2004, considered that the plant growth factors and conditions necessary for rooting varied among species, some studies reported strong effect of IBA in some recalcitrant species (HOLOBIUC et al., 2004; HUSAIN et al., 2008), or the positive effect of IAA comparing to IBA (ABBAS et al., 2012).

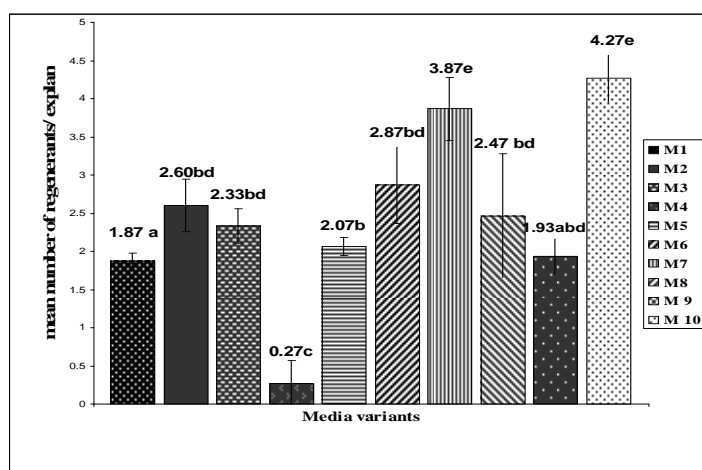


Figure 1. The evaluation of *in vitro* regeneration of *C. persicus* after 60 days of culture. Mean values with the same letter do not differ significantly at $P < 0.05$.

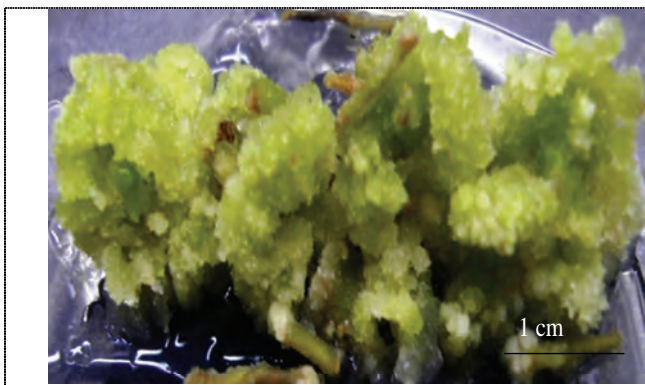


Figure 2. Proembryogenic friable callus induced from hypocotyls on M1 medium after 1 month (original).



Figure 3. The initiation of direct morphogenesis induced on root explants on M1 medium after 3 weeks (original).

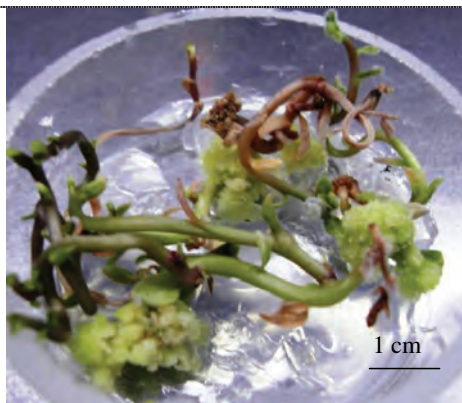


Figure 4. Indirect morphogenesis induced on M6 variant after 1 month (original).

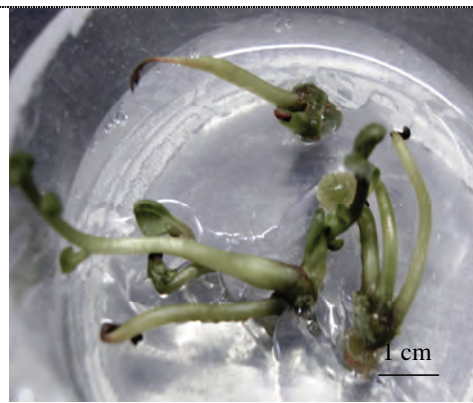


Figure 5. Direct morphogenesis on stem fragments induced after 30 days on M10 medium (original).



Figure 6. Rooted plants of *C. persicus* developed on M11 variant after 1 month (original).



Figure 7. *C. persicus* plants cultured on MS medium without growth factors with weak rooting (original).

Biochemical evaluation of the regenerants

The stability of individuals belonging to two different clones obtained through direct morphogenesis was evaluated using isoenzymes markers.

The use of biochemical markers to evaluate the identity of regenerants is more affordable and easily to perform comparing to molecular methods, but it is important to find the relevant biochemical markers for a certain plant species. Concerning the biochemical evaluation of the regenerants obtained through direct morphogenesis, in the case of regenerated clones (A and B), the isoenzymes spectra was expressed differently comparing to mature plants, probably due to *in vitro* conditions and age of plants.

The isoperoxidase spectrum (POX) analysed in individuals belonging to the same clone with the origin in one seed, shown the same number of bands, but with some differences in intensity (Fig. 8a).

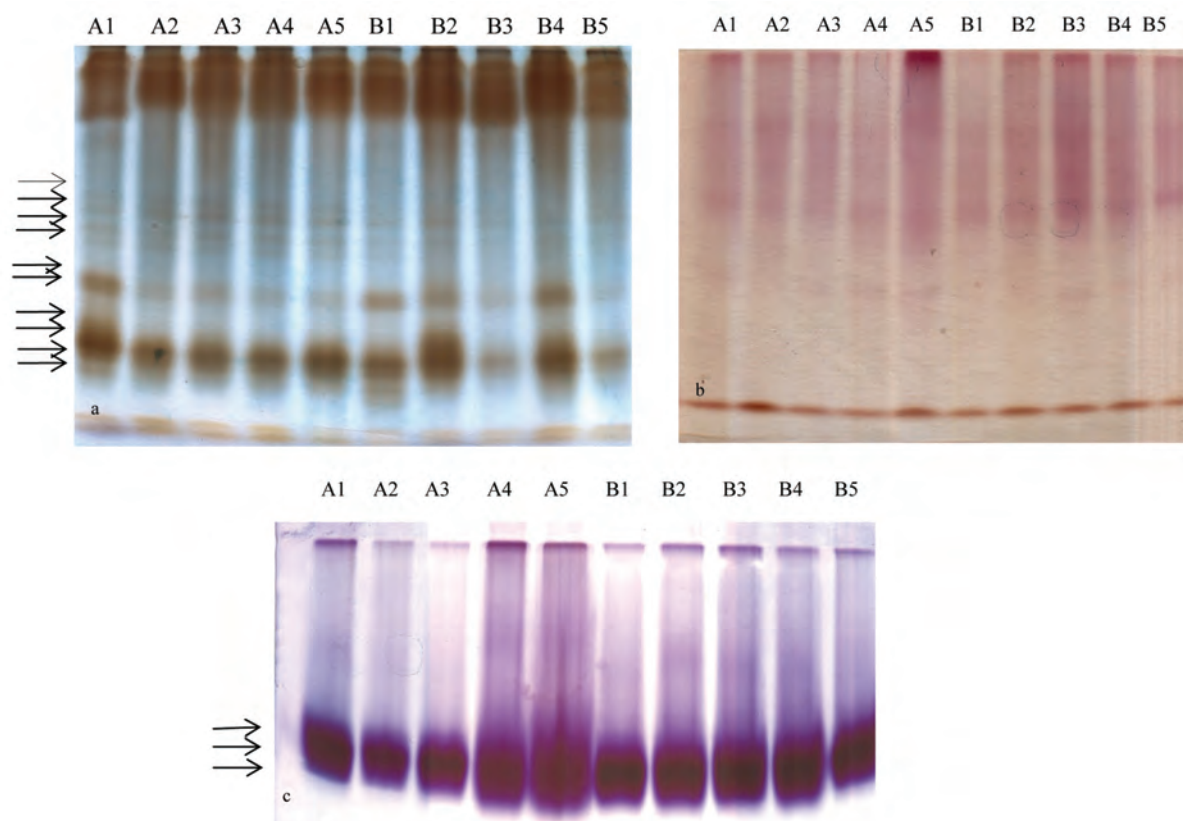


Figure 8. Electrophoresis spectra of isozymes from 2 clones (A, B) each of them consisting in ten individuals (A1-A5; B1-B5): peroxidases (a), esterases (b) and malate dehydrogenase (c).

In the case of clone A, A1 individual had more intense bands and for clone B, individuals B1, B2, B4 had also higher expression correlated with plants development. It is possible that the two *in vitro* regenerated clones have a certain level of genetic similarity concerning this marker.

In the case of catalase detection (CAT), in the analysed regenerants, there were not evidenced bands.

Its expression may be correlated with a certain developmental stage (it is expressed in mature lignified plants) and was not expressed *in vitro*. In the natural population, CAT expression was detected with a certain level of polymorphism (VOICHIȚĂ et al., 2013). A similar result was observed in the case of alkaline phosphatases (AKP); the bands were not evidenced in the regenerants.

Isoesterase spectrum (EST) did not show any differences among the individuals and even different clones (Fig. 8b). The expression of this enzyme probably was influenced by the culture condition and developmental stage of the regenerants, being not suitable as biochemical marker to check the regenerants identity in this taxon.

Malate dehydrogenase spectrum (MDH) was further analysed in the regenerants developed from direct morphogenesis and showed the same pattern of bands in the case of individuals from the same clone (Fig. 8c).

Several papers reported the characterization of intra-populational diversity in different taxa using biochemical markers. MÜLLER-STARCK et al., (1993) characterized the intra- and inter-populational genetic variation in *Quercus robur* and *Q. petraea* using more enzymatic systems to identify the genetic polymorphism as leucyl aminopeptidases, phosphoglucomutase, menadione reductase.

There were reported some relevant studies in which enzyme spectra was successfully used in the case of endangered or endemic plant species, mainly to characterize intra-populational variability (KRZAKOVA, 1996; ZHELEV et al., 2002; BORBA et al., 2007; KENNEDY et al., 2007; KULL & OJA, 2010).

KRZAKOVA (1996) based on isoperoxidase spectrum, found variation among individuals and populations in *Phragmites australis*.

BUTIUC-KEUL (2006) also described that the isoperoxidases pattern in *Puccinella poissoni* characterized small intrapopulational diversity, while the isoesterase had a relevant pattern concerning variability. By the other hand, the electrophoretic spectra of POX and EST analysed by the same author were found to be identically in all plants analysed from *Jurinea molis*.

In Romania, genetic polymorphism in the endemic species *Astragalus peterfi*, was also described using others biochemical markers as: alcohol-dehydrogenase, superoxide dismutase, and malate dehydrogenase (BORZA et al., 1996).

The genes, which are responsible for these enzymes, depend on the species, being necessary to carry out analyses to find the suitable marker. The enzyme detection, if they are appropriate for variability characterization could be more affordable.

In a previous study concerning to use of isoenzymes spectrum to detect variation among the individuals of *C. persicus*, just POX and CAT proved to be suitable for the evaluation of intra-population diversity, while esterases and phosphatases were not optimal for this purpose (VOICHIȚĂ et al., 2013).

By the other hand, in the case of *in vitro* regenerants, only peroxidase spectrum showed some differences, while the pattern of malate dehydrogenase and isoesterase were not relevant. It is possible that owing to *in vitro* culture conditions and developmental stage (young plantlets), the expression of the genes involved in this enzymes pattern to not be the same as in the natural population consisting in mature, flowering and lignified plants. Concerning *in vitro* culture, there were some reports based on the use of isoenzymes patterns to evaluate regenerants in different species mainly obtained through indirect morphogenesis (SAMANTARAY et al., 1999; PETROVA et al., 2006).

PETROVA et al. (2006) described in the case of regenerated plants of *Gentiana lutea*, significant differences in the expression of EST, ACPH and ADH, these results being rather correlated with the growth factors content, especially the cytokinin added on different culture media used to regenerate plants. This modified expression was considered as a result of a change of the expression of genes codifying loci responsible of these enzymes under the influence of growth regulators nature and their level used in vitro cultures.

In comparison, in our case, all regenerants analysed were obtained on M10 variant, further rooted on M11 medium, being influenced by the same combination of growth factors and culture conditions.

CONCLUSIONS

- *In vitro* developmental processes varied from the induction of non-regenerative callus and friable proembryogenic callus, to indirect and direct morphogenesis, depending on explants and media used.
- The variants of media with cytokinin dominance were optimal for shoots development starting from stem fragments, allowing a maximum of 5 shoots/explants. In the case of root fragments, morphogenesis can also occur, but with lower number of regenerants.
- Concerning rooting, Chu medium supplemented with active charcoal and low level of NAA sustained both a good rhizogenesis and stimulated the development of lateral shoots.
- The stability of individuals belonging to two different clones was evaluated using isoenzymes markers as peroxidase, malate-dehydrogenase and isoesterase, but only peroxidase showed a relevant spectrum of bands, the two analysed clones being related.

ACKNOWLEDGEMENTS

The research was supported financially by the Romanian Academy from project no. RO1567-IBB06/2011: 'Studies regarding *ex situ* conservation of natural gene pool for establishing a bank for plant tissues and cells culture for biotechnological and conservative purpose'.

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Received: March 31, 2015
Accepted: July 18, 2015