DIRECT SOMATIC EMBRYOGENESIS OF THE ENDEMIC TAXON *Papaver alpinum* L. SSP. *Corona-sancti-stefani* (ZAPAL.) BORZA FOR CONSERVATIVE PURPOSE

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Abstract. A protocol for regeneration through direct somatic embryogenesis in the endemic taxon *Papaver alpinum* L. ssp. *corona-sancti-stefani* (Zapal.) Borza was developed. Being both a rare and endemic plant species, this species requires a rescue action using tissue cultures. The somatic embryogenesis was induced from leaf and root explants cultured on MS medium supplemented with different concentrations of cytokinins (BA, kinetin), auxins (2.4-D and NAA) and supplementary additives (mannitol and sucrose). After 90 days of culture at 25ºC, clusters of somatic embryos were obtained. Somatic embryos were developed on all tested media, but the highest number (35 ± 2.43) was recorded on Murashige & Skoog medium supplemented with BA 1 mg/l, NAA 0.2 mg/l and kinetin 1 mg/l. The somatic embryos were converted into plants by transferring on the same medium variant (added with plant growth factors or mannitol) at 10°C. This is the first report of direct somatic embryogenesis in the case of *P. corona-sancti-stefani*.

Keywords: *P. corona-sanct-stefani*, somatic embryos, *ex situ* conservation, endemic.

Rezumat. Embriogeneza somatică directă la taxonul endemic *Papaver alpinum* L. ssp. *corona-sancti-stefani* în scop conservativ. În cazul taxonului endemic *Papaver alpinum* L. ssp. *corona-sancti-stefani* a fost realizat un protocol de regenerare prin embriogeneză somatică directă. Fiind o specie de plante rară şi endemică, necesită acţiuni de conservare utilizând tehnica culturilor de ţesuturi. Embriogeneza somatică a fost indusă pornind de la explante reprezentate de fragmente de frunze şi rădăcini. Acestea au fost cultivate pe mediu MS (Murashige & Skoog) suplimentat cu diferite concentraţii de citokinine (BA, Kinetin), auxine (2,4-D şi NAA) şi aditivi suplimentari (manitol şi zaharoză). După 90 de zile de cultură la temperatura de 25°C, s-au obţinut clustere de embrioni somatici. Embrionii somatici s-au dezvoltat pe toate variantele de medii testate, dar cel mai mare număr de embrioni (35 ± 2,43) a fost înregistrat pe varianta de mediu suplimentat cu BAP 1 mg/l, ANA 0,2 mg/l şi kinetin 1mg/l. Embrionii somatici au putut fi convertiţi în plante prin transferul lor pe aceeaşi variantă de mediu (adăugat cu factori de creştere sau manitol), dar la temperatura de 10°C. Acesta este primul raport de embriogeneză somatică directă semnalat în cazul speciei endemice *P. corona-sancti-stefani.*

Cuvinte cheie: *P. corona-sancti-stefani*, embrioni somatici, conservare *ex situ*, endemit.

INTRODUCTION

Papaver alpinum L. ssp. *corona-sancti-stefani* (Zapal.) Borza (Papaveraceae family) is the unique representative of the genus in the Romanian alpine vegetation and has an important role as pioneer plant in the substrate formation by fixing the detritus (DIHORU & PÂRVU, 1987).

Being an endemic (CIOCĂRLAN, 2009) and rare (OLTEAN et al., 1994) plant species in S-E Carpathian Mountains, it is necessary to adopt suitable rescue actions for the conservation programs (IŞIK, 2011), which can involve both *in situ* and *ex-situ* conservation. A secure *in situ* conservation is the first requirement to preserve the endangered plant species, but in the case of deterioration of the natural environment, the *ex situ* conservation is recommended. The *in vitro* techniques play an important role in the *ex situ* conservation programs, being the most efficient way to propagate a species without depleting wild resources (REED, 2011).

In vitro somatic embryogenesis as a plant regeneration way has the potential to produce a large number of plantlets, which may be used for conservation programs, for the reintroduction in natural habitats, as well as for utilization in basic research, etc.

In the present study, our aim was to develop a reliable protocol for *in vitro* regeneration of the endemic *Papaver alpinum* L. ssp. *corona-sancti-stefani* (Zapal.) Borza through direct embryogenesis for conservative purpose.

MATERIALS AND METHODS

Plant material comprised of mature seeds was collected from natural population from the northeast part of Piatra Craiului (N: 45°31.307 E: 025°11.849), slope 60°, in the year 2007. The seeds were kept in a paper bag at the room temperature.

Sterilization protocol: In the first step of the protocol, the seeds were washed with tap water for 2 hours and surface-sterilized in 70 % ethanol for 30 seconds followed by 10 minutes immersion in a 0.01% $HgCl_2$ and finally washed three times with sterile distilled water.

A total of 100 seeds were checked for the germination capacity. The seeds were placed on the MS media variant (MURASHIGE & SKOOG, 1962) added with 30g/l sucrose and B_5 vitamins (GAMBORG et al., 1968), without plant growth factors.

***In vitro* cultures** were performed from the seedlings. The explants (leaves and root segments) were placed on the MS media with 3% (w/v) sucrose supplemented with different concentrations of plant growth factors and additives

(Table 1). The media cultures were solidified with Plant agar (Duchefa) 0.8% (w/v) and the pH was adjusted to 5.8. The photoperiod was 16h light/8h dark with light intensity of 27µmol*m^{-2}*sec^{-1}, at temperature 24±2°.

The mean number of somatic embryos/explant was used like parameter to quantify the regeneration efficiency. Three replicates with 5 explants/Petri dish were cultured. One-way analysis of variance was used to calculate the statistical significance (http://xltoolbox.sourceforge.net).

Table 1. The media variants tested in *P. corona-sancti-stefani*.

		Media variants				
		MO	**M1**	**M2**	**M3**	**M4**
Plant growth factors (mg/L)	BA	-	1	-	-	-
	NAA	-	0.2	-	-	-
	2.4 D	-	-	0.2	-	-
	KIN	-	0.1	0.1	-	-
Additives (g/L)	Mannitol	-	-	-	30	-
	Sucrose	-	-	-	-	30

Legend: BA – N^6-benzylaminopurine, NAA – α-naphthalene acetic acid, 2,4-D – 2,4 dichlorophenoxyacetic acid; Kin – kinetin.

RESULTS

The sterilization of seeds has an important role to ensure plant material for *in vitro* procedures. In our case, no contamination was observed. Seeds started to germinate after 45 days but the germination rate was low (CATANĂ et al., 2013).

As first response, the explants (leave and root fragments) started to become brown and degenerated. After 60 days early stages of somatic embryogenesis was detected (Fig. 1).

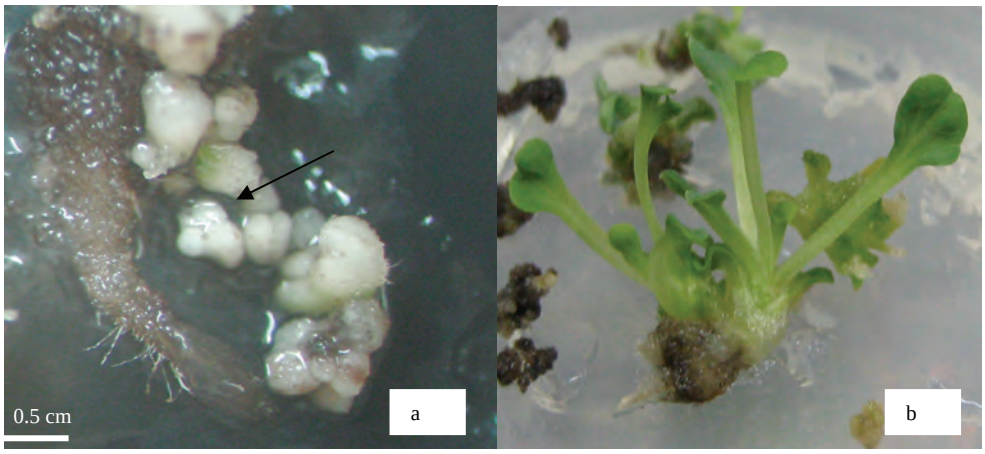

Figure 1. Different early stages of somatic embryogenesis starting from roots as explant (a) and embryos converted into plants (b) on the M1 variant (BA, NAA, kin) (Original photos).

The regenerants number/explant varied between 6 and 35 depending on the variant. The best result was obtained on the MS supplemented with 1 mg/l BA, 1mg/l Kinetin and 0.2 mg/l NAA (M1 variant) (Table 2).

Table 2. Regeneration and embryo conversion on different media variant tested.

	No. of regenerants /explant	**% conversion of somatic embryos into plants**	**Observations**
M0	$0^{a,d,g}$	39.13%	Ensure somatic embryos germination
M1	$35±2.43^{c,f,g}$	76.92%	Somatic embryos are converted into plants; favour the rooting process
M2	$25±3.14^{d,e,f}$	10%	Somatic embryogenesis occur with low rate, most of somatic embryos failed to develop; low number of regenerants were obtained
M3	$30±1.38^{a,b,c}$	60.86%	Somatic embryogenesis was induced, underwent all stages germinated and converted into plants
M4	$5±1.01^{b,e}$	30%	Somatic embryogenesis was induced, part of them were arrested in early stages

Legend: *Values are represented as mean ± standard error (SE) of three independent replications. The values followed by the same superscript letters are statistically different (P≤0.05) from each other (Tukey Comparisons Test).

All stages of somatic embryos (from globular to cotyledonary embryos) were observed on the surface of explants (Fig. 3).

During the initial phases, the somatic embryos require a temperature of 24±2°C. The conversion was achieved by subculture on the media variants with and without plant growth factors at 10°C. In the M0 (control) variant, embryos conversion occurred but was comparatively slower than M1 variant (with plant growth factors). The M1 variant (supplemented with BA, NAA and kinetin) sustained the induction, the development and the rooting of the embryos.

When the sucrose concentration was increased at 60g/l (M4 variant), the embryos were induced, but the mean number was lower (5±1.014). The maintenance of the somatic embryos on the M4 variant (added with 60 g/l sucrose) more than 60 days with two subcultures conducted to the degeneration of the embryos (Fig. 2).

Figure 2. Somatic embryogenesis on the M4 medium variant (60g/l sucrose) (Original photos).

Adding 30g/l mannitol (M3 variant) a significantly increasing of the mean number of embryos/explant compared with M4 variant supplemented with 60 g/l sucrose (Fig. 3) was observed. The embryos were developed on the M3 variant were able to convert into plants.

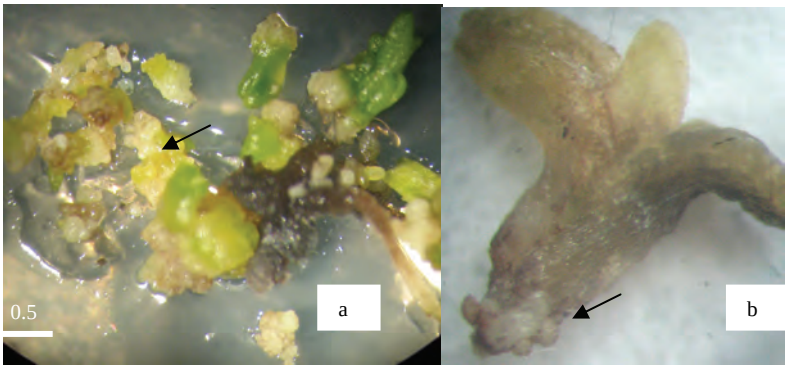

Figure 3. Direct somatic embryos induced on the M3 medium variant (30g/l mannitol): a) cluster of somatic embryos in different stages of development, b) embryo in cotyledonary stage with supplementary embryos attached (Original photos).

The plants developed through somatic embryos conversion maintained at 24±2°C and 10°C for more than 90 days showed some morphological differences concerning height, colour, root development and etiolating (Table 3, Fig. 4).

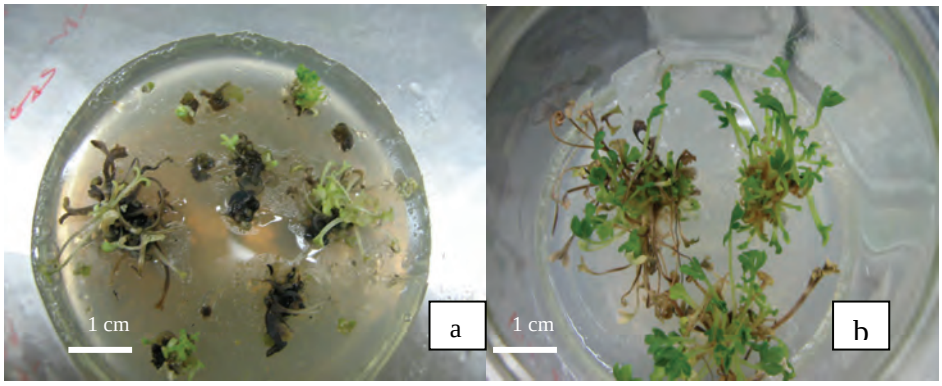

Figure 4. *In vitro* regenerants of *P. corona- sancti- stefani* maintained on the medium variant with plant growth factors at a) 25°C and b)10°C (Original photos).

Table 3. Morphological differences among the plants converted from somatic embryos maintained at different temperature (10°C and 25°C).

	10°C	**25°C**
Height	3 cm	0.7 - 2 cm
Colour	green	yellow pale
Root development	yes	no
Etiolation	no	yes

DISSCUSIONS

Most of the studies concerning *in vitro* culture in *Papaver* L. genus have been focused on the medicinal species concerning the alkaloid biosynthesis. *In vitro* cultures were used to induced callus (SARIN, 2003; AVIJEET et al., 2010; ZAKARIA et al., 2011; BONDARIAN et al., 2013), secondary somatic embryogenesis and shoot organogenesis from primary somatic embryos (NESSLER, 1982; OVEČKA et al., 1996, 2000; YANG et al., 2010; PATHAK et al., 2012). Concerning the endemic species, *in vitro* cultures were used in the case of glacial relict *P. degenii* (GORGOROV et al., 2011), where plants cultivated on MS solid medium without plant growth factors had a propagation coefficient of 2.4 for four weeks of cultivation.

The embryogenic competence is expressed at the level of the cells, which are able to change their fate and to have active divisions if they receive inductors for differentiation (FEHÉR, 2005). There are considered 2 categories of somatic embryogenesis inductors: internal and/or external cellular levels of plant growth regulators and stress factors (as physical or chemical - osmotic shock, culture medium dehydration, pH, heavy metal ions, heat or cold shock treatments, hypoxia, antibiotics, ultraviolet radiation, wounding treatments) (ZAVATTIERI et al., 2010).

In the case of *P. somniferum* L., callus was obtained starting from seedlings hypocotyls in the presence of 0.25 mg/l kinetin and 2.0 mg/l NAA (NESSLER, 1982). The same combination of plant growth factors but in different concentration was used for the induction of callus in *P. somniferum ssp. album* and *P. orientale ssp. splendidissimum* (KASSEM & JACQUIN, 2001). In the case of *P. nudicaule,* an ornamental medicinal plant, a regeneration way through somatic embryogenesis and secondary somatic embryogenesis on MS medium containing 1.0 mg/L NAA and 0.1 mg/L BA starting from petiole explants was reported (YANG et al., 2010).

The maturation stage is considered a critical stage of somatic embryogenesis and depends on the presence of specific plant growth regulators (MONDAL et al., 2002). As in our case, the embryos from different stages were converted into plants on the same medium variant, the embryos of *P. nudicaule* germinated with high frequency on the medium supplemented with BA and NAA (YANG et al., 2010).

Our results showed that mannitol and sucrose are able to induce somatic embryogenesis, but only mannitol can sustain the development of the embryos. Similarly, mannitol was proved to induce somatic embryogenesis in some taxa as *Dianthus spiculifolius*, *D. tenuifolius*, *D. glacialis* ssp *gelidus* (HOLOBIUC & CATANĂ, 2012), G*entiana lutea* (HOLOBIUC et al., 2010).

In our case, in the presence of 6% sucrose, somatic embryos were arrested in the early stages and they did not evolve and was observed different stages of degradation (Fig. 2). The same results were obtained in the case of *Papaver somniferum ssp. album* and *P. orientale* ssp. *splendidissimum*, where increased sucrose concentration did not allow the development of somatic proembryos to somatic embryos (KASSEM & JACQUIN, 2001).

CONCLUSIONS

In the case of *P. corona-sancti-stefani*, a protocol of micropropagation through direct somatic embryogenesis was established. As inductors of somatic embryogenesis in our case, there can be used plant growth factors (as BA, NAA and kinetin) and also mannitol as stress factor. The maturation of the embryos was achieved in good conditions on the medium variant with plant growth factors at 10°C.

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