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LEONTOPODIUM NIVALE SSP. *ALPINUM* (CASS.) BIOTECHNOLOGY APPLIED FOR ECONOMIC AND CONSERVATIVE PURPOSE

Mihaela Irina HOLOBIUC, Alexandra-Gabriela CIOCAN, Rodica CATANĂ

Institute of Biology Bucharest of Romanian Academy, 296 Splaiul Independentei, 060031, District 6, P.O. Box 56-53, Bucharest, Romania

Corresponding author email: alexandra.ciocan@ibiol.ro

Abstract

Plant biotechnology ensures the production of a wide range of compounds for industry, pharmacy, cosmetics and medicine. Tissue cultures can provide useful metabolites all-round the year under controlled conditions with reduced expenses. L. nivale subsp. alpinum (Cass.) is a protected taxon, of ornamental value and economic importance.
Our aim was to produce highly regenerative cultures for mass production and synthetic seeds development, as mean for multiplication and preservation.
The effect of different growth factors was studied concerning regeneration through direct morphogenesis and somatic embryogenesis. The presence of 2.4-D and Dicamba alone or combined with low levels of kinetin favoured somatic embryogenesis. Histological samples of embryogenic aggregates were analysed using optical and scanning electron microscopy. Somatic embryogenesis provides a large mass of plant material for basic studies and for economic purposes.
For synthetic seeds production, embryogenic aggregates of 2-3 mm diameter were incubated in 3% sodium alginate in a modified $CaCl_2$-free MS liquid medium. Subsequently, dehydrated seeds were cultured on MS agar solidified medium added with growth regulators to test the artificial seeds' growth.
We improved the regeneration rate through somatic embryogenesis and, subsequently, produced artificial seeds useful for multiplication and conservative purposes.

Key words*: Leontopodium nivale, somatic embryogenesis, synthetic seeds.*

INTRODUCTION

L. nivale ssp. *alpinum* (syn. *L. alpinum*) is a perennial alpine herbaceous, hemicryptophyte taxon known as Edelweiss, growing on rocky substrates as limestones in different European alpine area as in the Alps, Pirini, Rhodopes and Carpathian Mountains. This taxon is threatened in several European countries and populations in the past declined due to extensive collection; having an extent distribution and lacking information on population reduction, it is listed as Least Concern (Khela, 2013). *L. nivale* flowers are highly prized and have been collected from the wild (Pace et al., 2009), a practice that is now regulated or banned in many European countries (Kozuharova et al., 2018).

This alpine plant became of great interest in the latest years for pharmaceutics and cosmetics industry, owing to its valuable compounds with biological activities (Tauchen & Kokoska, 2016). Specifically, different classes of compounds such as terpenoids (analogues of sesquiterpenes, bisabolenes), phenylpropanoids (phenolic acids, flavonoids, coumarins, lignans), fatty acids and polyacetylenes, extracts and compounds have been characterized and found to have a broad spectrum of pharmacological activities on the cardiovascular and nervous system, but also anti-inflammatory, antimicrobial, antioxidant and chemo-protective effects.

The Edelweiss extract proved to be a valuable constituent of anti-aging skin treatments, being included in a wide number of dermo-cosmetical products already existing on the market, due to its antioxidant and anti-inflammatory effects (Campiche et al., 2022). Owing to the large economic and industrial demand for compounds and Edelweiss' protected status in many countries, alternative methods were developed, of which the biotechnological approach proved to be reliable and affordable (Pralea et al., 2021).

Small scale *ex situ* cultivation (Kozuharova et al., 2018) and *in vitro* biotechnological approaches are economically viable alternatives to the production via chemical synthesis of useful compounds (Ciocan et al., 2023).

Biotechnology has the potential for large-scale plant production in horticulture, forestry and medicinal plants.

The elaboration of highly reproducible, efficient micropropagation protocols provides continuous plant production (Anis & Ahmad, 2016) and sustains the germplasm preservation in a tissue bank, ensuring material for slow-growth culture and cryopreservation (Kulus, 2016). Plant tissue cultures can provide high-quality planting material throughout the year, irrespective of season and weather (Shahzad et al., 2017).

In vitro seed germination, vegetative propagation and acclimatization methods contribute to propagule production, both for preservation strategies and for translocation or reintroduction projects (Chauhan, 2016).

In the case of endangered species, the process might require modified, improved or combined strategies for *ex situ* preservation (Sarasan et al., 2006; Reed et al., 2011; Hurdu et al., 2022).

Micropropagation represents an alternative to overcome population decline in the natural habitat due to climatic changes and antropization and also the establishment of valuable metabolite-producing cell lines (Fasciani et al., 2011).

The biotechnological approach generally targets three main directions: (1) the production and optimization of callus and suspension cultures for useful secondary metabolites' production, including different ways of stimulation or elicitation; (2) the production of hairy roots after genetic transformation (Wawrosch et al., 2014) and (3) *in vitro* micropropagation and the production of secondary metabolites (Hook, 1994). In our work, we aimed to improve the *in vitro* production of *L. nivale* ssp. *alpinum* plant material useful for ornamental and economic purposes, starting from aseptically germinated seeds, induction and evaluation of the micropropagation through direct morphogenesis and somatic embryogenesis, along with the characterization of somatic embryogenesis, as an optimal mean to multiply and to preserve this taxon through synthetic seeds production.

MATERIALS AND METHODS

Seed sterilization

Commercial seeds (Germany) were sterilized following a three step protocol: (a) a two-hour wash in running tap water; (b) short immersion in 70^0 ethanol, followed by a treatment with mercuric chloride 0.1% (12 minutes) and (c) three washes with sterile distilled water. The sterilized seeds were cultivated on MS (Murashige & Skoog, 1962) agar solidified (8 g/l) medium, added with 20 g/l sucrose, cultured 6-10 seeds/ Petri dish.

In vitro culture initiation

Seedlings germinated after 7-10 days and were used as explants for establishing *in vitro* cultures. Different media variants were tested, based on the MS formula (Murashige, Skoog, 1962) and supplemented with Gamborg B5 vitamins (Gamborg et al., 1968), 30 g/l sucrose, 7 g/ l agar (Duchefa Biochemie), pH adjusted to 5.8 and additional compounds (Table 1).

The subcultures were performed every 4 weeks. Cultures were maintained in the growth chamber with a light intensity of 55 µECmu-$2s^{-1}$ at 25°C and a photoperiod of 16 light /8 h darkness.

Direct morphogenesis

To induce the morphogenetic response, 5 seedlings were inoculated in 6 cm/10 cm high glass jars in 3 replicates/variant, on media based on the MS formula added with 30 g/l sucrose, Gamborg vitamins and different plant regulators (Table 1). After regeneration, 9-10 shoots were further cultivated in autoclavable polypropylene dishes (9x10x10 cm) on Murashige Skoog medium, supplemented with 20 g/l sucrose, 0.5 g/l charcoal and solidified with 8 g/l Duschefa agar.

Callus and somatic embryogenesis induction

For the callus and somatic embryogenesis induction, fragments of *in vitro* regenerated seedlings (roots and leaves) were used as explants. Fragments of about 5 mm long were cultured on 6 cm diameter Petri dishes, culturing five explants x 3 repetitions/medium variant. Several media variants based on the MS formula (Murashige & Skoog, 1962) added with 30 g/l sucrose and B5 vitamins according Gamborg formula were tested (Table 2). After one month, embryogenic calli of 1 cm diameter were subcultured on MS 1/2 hormone free medium supplemented with 500 mg/l charcoal,

20 g/l sucrose, solidified with 8 g/l Duschefa agar, at pH 5.8, to ensure embryos' development.

Table 1. Media variants tested for direct morphogenesis

Media	Growth factors (µM)				Other compounds (mg/L)		
	BAP	Kin	Zea	NAA	Glut	$CaCO_3$	Ch
MSL 1	4.44	-	-	0.18	-	-	-
MSL 2	-	4.6	-	0.18	-	-	-
MSL 3	4.44	4.6	-	0.90	-	-	-
MSL 4	4.44	4.6	-	0.36	-	-	-
MSL 5	4.44	4.6	-	0.36	-	100	-
MSL 6	4.44	4.6	-	0.36	250	-	-
MSL 7	4.44	4.6	-	0.36	-	-	300
MSL 8	-	-	4.56	0.18	-	-	-

Legend: 6-BAP - 6-benzylaminopurine; Kin - kinetin; Zea - zeatin; NAA - 1-naphthaleneacetic acid; IAA - indole-3-acetic acid; Glut - glutamine; Ch- casein hydrolisate

Table 2. Media variants tested for callus and somatic embryogenesis induction

Variants	Growth factors (µM)				
	2,4 D	Dic	IBA	NAA	Kin
MSL9	-	2,26	-	-	-
MSL10	2.26	-	-	-	
MSL11	-	4.52	4.9	-	0.92
MSL12	4.52	-	4.9	-	0.92
MSL13	-	2.26	-	-	0.92
MSL14	2.26	-	-	-	0.92
MSL15	-	4.52	-	-	0.92
MSL16	4.52	-	-	-	0.92
MSL17	9.04	-	-	-	4.6
MSL18	4.52	-	-	-	9.2
MSL19	-	-	-	1.8	9.2
MSL20	-	-	-	3.6	4.6

Legend: Kin - kinetin; 2,4-D - 2,4-dichlorophenoxyacetic acid; Dic- Dicamba; IBA - indole butyric acid

In vitro culture characterization

The efficiency of plant production via direct morphogenesis was evaluated taking into account the mean number of regenerants/initial inoculum and the maximum shoots length (cm) after two months.

The effect of different combinations of growth factors was analysed regarding callus production and regeneration through morphogenesis and somatic embryogenesis. Somatic embryogenesis was evaluated taking into account the explant's source (leaves and roots). The rate of callusing of initial explants (the number of calli developed/ initial explant x100) was evaluated in the first month of culture.

The rate of regenerative calli (the number of regenerated calli/total number of cultured calli x100) and the mean number of regenerants/ explant were recorded for each experimental variant after two months. Ten initial explants were taken into consideration for each variant for somatic embryos evaluation.

Microscopical analysis

Optical microscope

For the histological observations, the aggregates of embryogenic cultures were prepared in several stages: (a) fixation in formaldehyde 3.7%; (b) serial dehydration in ethanol for 15 minutes (70, 80, 96°); (c) 6-hour immersion in butyl alcohol; (d) immersion of the samples in benzene 2 times x 15 minutes and (e) embedding in paraffin at 60° for 2 hours. Paraffin-embedded sections were sectioned using an LKB microtome at 7 µm. For removal of paraffin, the sections were immersed in xylene for 30 minutes, transferred in alcohol series 96-15° for 15 minutes, washed and stained in Hemalaun Mayer for 10 minutes, followed by washing in running tap water.

For stereomicroscopy analysis, embryogenic cultures were visualized under a binocular microscope Stemi-2000C Zeiss. For optical microscope analysis, fresh embryogenic cultures were stained with 0.1% methylene blue dissolved in a drop of distilled water. The samples were prepared by spreading the plant material on a slide and covered with glass.

For scanning electron microscopy (SEM), embryogenic aggregates were fixed in 3% glutaraldehyde adjusted to pH 6.8 in 0.1 M cacodylate phosphate buffer for 24 h at 4 °C. The tissue was washed in the same buffer, post fixated for 2 h in a similarly buffered 1% osmium tetroxide solution, dehydrated in ethanol series (50, 70, 90, 100°) for 10 minutes, air dehydrated for 130 minutes and finally mounted on slide and coated with gold nanoparticles. The prepared samples were examined and photographed using a scanning electron microscope Jeol JSM 6610LV and 20 kV acceleration.

Synthetic seeds production

Fragments of embryogenic aggregates of 2-3 mm diameter were incubated in 3% sodium alginate (10 min) in a modified $CaCl_2$-free MS liquid medium supplemented with 87 mM sucrose and sterilized by autoclaving. Using a cut pipette tip (5 mm diameter), they were gently dropped into liquid MS supplemented with 0.1 M $CaCl_2$ and left for 30 min. The obtained artificial seeds were washed with sterile distilled water and osmotically dehydrated in

100 ml liquid MS medium, with gradually increased sucrose concentrations: 0.25 M (24 h), 0.50 M (24 h), 0.75 (24 h), being kept in agitation using an orbital shaker at 130 rpm, in the dark. Sterile synthetic seeds were kept one week at 4°C. Subsequently, seeds were cultured on MSL9 or MSL10 agar solidified medium to allow further development.

Statistical analysis

The experimental data obtained were checked for normality, using Shapiro-Wilk's test and homogeneity of variance, using Levene's test. Afterward, they were subjected to a one-way analysis of variance (ANOVA), to assess the statistical significance, followed by a Tukey HSD test to determine the differences between means. All analyses were performed using the software R 4.4.1 (R Foundation for Statistical Computing, Austria) and Origin, Version 2024 (OriginLab Corporation, Northampton, MA, USA). Error bars of graphics represent the standard deviation of mean value (±SD) and *p*-values $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) were considered statistically significant.

RESULTS AND DISCUSSIONS

Seed germination

The germination of seeds started after 7-10 days. The registered rate of sterilization was 80% and a maximum germination rate of 70 % was obtained after two weeks.

After the initiation of aseptic tissue cultures, plant regeneration was conducted through two main developmental processes: morphogenesis and somatic embryogenesis, based on plant cell totipotency.

In the morphogenetic process, developed roots and shoots establish vascular connections with the tissues of the origin explant, while in the case of somatic embryos (Se), no vascular connections with the explant tissues are present, being similar to zygotic embryos, with a bipolar structure, but characterized by the absence of developmental arrest and the endosperm presence (Faure et al., 1998).

Direct morphogenesis

In vitro culture evaluation

The mean number of shoots/inoculum varied between 5(on the MSL3 medium variant (Figure 3 a) and 32 (on MSL6), the values being significantly different ($p < 0.001$) among tested media (Figure 1).

Concerning the maximum length of regenerated shoots, the values varied between 1.5 cm for shoots regenerated on MSL1 and 2.6 cm for shoots regenerated on MSL3, with significant differences ($p < 0.001$) between media variants (Figure 2).

The addition of benzyl amino purine combined with kinetin and alfa naphthyl acetic acid, in presence of glutamine proved to be more beneficial and improved the regeneration rate through direct morphogenesis, with a maximum of 38 shoots/initial explants recorded (Figure 3 b).

Also, regeneration was stimulated by zeatin added in the MSL8 variant, however shoots growth was slower.

Neo shoots further developed on the primary shoots, increasing the number of regenerants after two months. The shoots rooted easily, but required to be detached and further cultivated on MS hormone-free medium for growth (Figure 3 c), the entire multiplication cycle taking more than three months.

The plants can be acclimated when necessary using the successful protocol described by Kotzuharova et al (2018).

In *L. nivale*, Hook (1994) first reported *in vitro* regeneration through shooting on apical buds and seedling explants cultured on MS medium supplemented with low levels of hormones, but the regeneration rate was not registered. Callus was also induced on MS medium added with 0.22 mg/l 2.4-D and 0.18 NAA, next used for suspension cultures establishment.

In the same taxon, Pace et al. (2009) succeeded in inducing callus from cotyledons from *in vitro* germinated seeds, but on Hoagland medium (Hoagland & Arnon, 1938) supplemented with 2,4-dichlorophenoxyacetic acid.

The regeneration of shoots occurred after the transfer of callus on a medium supplemented with 0.25 mg/L NAA and 0.50 mg/L6-BAP.

In this report, through indirect morphogenesis, over 30 shoots per callus were obtained and rooting was possible in the presence of auxin.

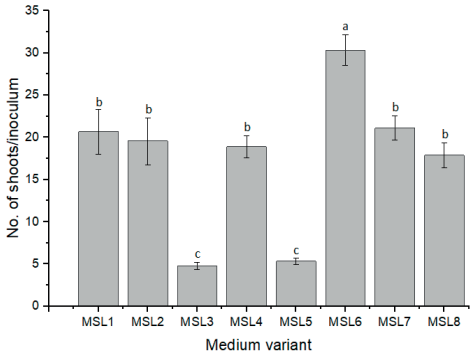

Figure 1. The effect of different medium variants on the number of shoots/inoculum. The data were recorded at the end of the experiment and are presented as mean ± SD. Values with different letters are significantly different ($p < 0.001$), using ANOVA and Multiple pairwise-comparison Tukey test (n= 9)

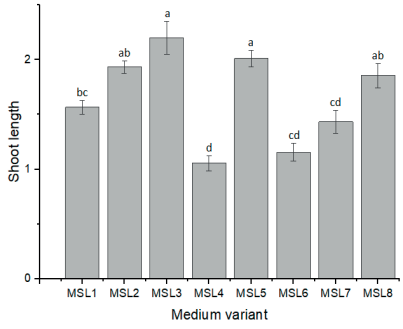

Figure 2. The effect of different medium variants on the maximum shoot length (cm). The data were recorded at the end of the experiment and are presented as mean ± SD. Values with different letters are significantly different ($p < 0.001$), using ANOVA and Multiple pairwise-comparison Tukey test (n = 9)

Using shoot tips, fragments of cotyledons and hypocotyls excised from 10-day-old seedlings, Trejgell et al. (2010) also reported the highest frequency of axillary shoot proliferation on shoot tip explants, the average of 15.1 shoots/ explant being obtained on medium supplemented with 4.4 µM BAP and 0.54 µM NAA.
Vasile et al. (2011) also reported a good regeneration in *L. alpinum* on the medium Adenin sulphate, with a high number of regenerants, but without specifying the duration of cultures and number of passages. In our experiment, the multiplication rate through direct morphogenesis, starting from aseptically germinated seeds, increased in subsequent stages, reaching over 30 regenerants/initial explant (maximum 38) after two months of culture on variant ML6, supplemented with BAP, NAA and glutamine (Figure 3b).
On the other hand, variants MSL1, MSL2, MSL7 and MSL8 ensured an acceptable regeneration.

Figure 3 a, b, c. Aspects of direct morphogenesis in *L. nivale* (scale bar = 1 cm)

The rooting occurred easily without additional treatments, but the transfer of mini-plantlets on large Phytatray vessels with MS medium added with charcoal, improved growth (Figure 3 c).

Somatic embryogenesis

Somatic embryogenesis is a multistep process, starting with proembryogenic masses that progress to the next stages maintaining the bipolar pattern of cell. Auxins and cytokinins are essential to maintain proembryogenic proliferation (Xu et al., 2013).
The process was induced as a result of auxin presence or dominance in the culture medium.
The dedifferentiation and redifferentiation occurred directly on the explant surface, callus cells also appeared (Figure 4 a, b), followed by embryogenic stages (Figure 5 b).

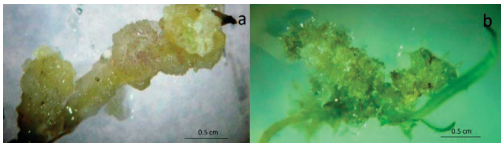

Figure 4. The induction of callus on MSL9 variant starting from root explant (a) and leaves (b)

Firstly, proembryogenic callus developed (Figure 5 b), followed by embryogenic stages (Figure 5 c).
Generally, the variants supplemented with 2.4-D and Dicamba ensured a good callusing rate, varying between 90 and 100% (Table 3). The low auxin level supplemented media variants (MSL9 and MSL10) induced a good response, while the variants containing low levels of auxins and kinetin also determined a positive embryogenetic response, somatic embryos evolving even on hormone added media.

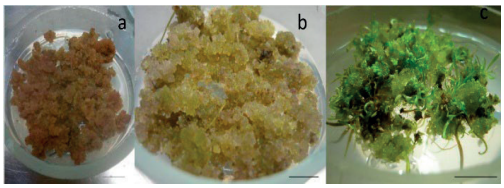

Figure 5. The effect of different media variants *in L. nivale*: a - nonregenerative callus; b - proembryogenic friable callus; c - high embryogenic cultures on MSL13

The number of recorded regenerants was over 50/initial explant. The ratio 2/1 of auxins (2.4-D or Dicamba combined with IBA) in MSL11 and MSL12 led to a high proembryogenic mass production with a lower rate of somatic embryos' conversion, therefore a transfer on hormone free MS medium was necessary.

Auxin treatment of explants was reported to be an indispensable inducer of somatic embryogenesis (SE) in a large number of plant species (Wójcik et al., 2020).

When NAA and kinetin were present in the growth medium, the callusing rate decreased and a nonregenerative callus was obtained (Figure 5 a) (Table 3). On the other hand, the higher level of kinetin combined with NAA did not have a positive impact on callogenesis, conducting to the browning of the culture.

On low auxin added variants (MSL 9, 10, 15, 16), all developmental stages of somatic embryos occurred from earlier to the later stages, even converting directly into plants.

On MSL11-14 variants, proembryogenic and early embryogenic stages developed, however, plantlet formation was favoured after the transfer on MS hormone free medium added with 0.5 g/l active charcoal (Figure 7 a, b).

The number of somatic embryos varied between 20 to over 50/initial explant after 8 weeks of culture, which was higher than the number of regenerants recorded through direct morphogenesis.

Considering the presence of root meristems, the developmental process of these structures was easier, faster and subculturing could be performed monthly, the rate of plant production being improved through somatic embryogenesis, compared to morphogenesis or shooting (Figure 7).

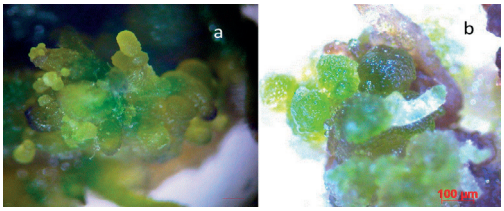

Figure 6. Somatic embryos developed on leaf (a) and root (b) explant on MSL 13 medium

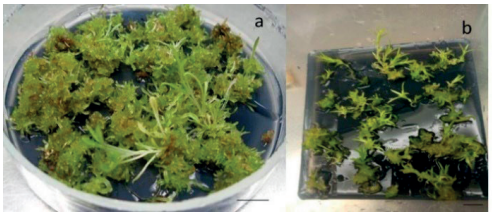

Figure 7. Highly regenerative cultures and plant development on MS medium supplemented with active charcoal (scale bar = 1 cm)

Table 3. *In vitro* response concerning callusing and somatic embryogenesis

Variants	Explant type	In vitro response	% of callusing	% of regeneration	Mean number of regenerants/ explant ± SD
MSL9	Root	Brown yellow embryogenic callus	80-100%	83.3%	21.1 ± 5.44^{b}
	leaf	Greenish friable embryogenic callus	100%	100 %	$\mathbf{54.9 \pm 8.54}^{t}$
MSL10	Root	Friable embryogenic callus	100%	90 %	19.8 ± 4.96^{b}
	leaf	Friable embryogenic callus	100%	100%	$\mathbf{56.2 \pm 15.75}^{t}$
MSL11	Root	Friable embryogenic callus	80-100 %	60%	11.5 ± 2.36^{c}
	leaf	Friable embryogenic callus	80-100%	50-60%	12 ± 3.26^{z}
MSL12	Root	Friable embryogenic callus	100%	60%	10.4 ± 2.75^{c}
	leaf	Green embryogenic callus	100%	50-60%	13.1 ± 3.90^{z}
MSL13	Root	Embryogenic callus	80-100%	80 %	20.9 ± 4.70^{b}
	leaf	Embryogenic callus	80-100%	80-100%	26.3 ± 4.87^{y}
MSL14	Root	embryogenic callus	80-100%	80%-100%	19.6 ± 3.53^{b}
	leaf	Embryogenic callus	80-100%	83%	29.2 ± 4.23^{y}
MSL15	Root	Embryogenic callus	80-100%	80%	36.3 ± 8.76^{a}
	leaf		80-100%	90-100%-	$\mathbf{45.6 \pm 7.98}^{tx}$
MSL16	Root	Embryogenic callus	80-100%	90-100%	38.2 ± 8.39^{a}
	leaf		80-100%	90-100%	$\mathbf{36.2 \pm 6.14}^{xy}$

Variants	Explant type	In vitro response	% of callusing	% of regeneration	Mean number of regenerants/ explant ± SD
MSL17	Root	Nonregenerative yellowish callus	80%	-	-
	leaf		80%	-	-
MSL18	Root	Yellow friable non-regenerative callus	60-70%	-	-
	leaf		80%	-	-
MSL19	Root	Brown non regenerative calli	60%	-	-
	leaf	Yellow Brown non regenerative callus	50-60%	-	-
MSL20	Root	Friable non regenerative callus	80%	-	-
	leaf	Friable non regenerative callus	100%	-	-

Values with different letters are significantly different ($p < 0.001$), using ANOVA and Multiple pairwise-comparison Tukey test

The positive effect of activated charcoal on embryo conversion was probably due to the adsorption of growth inhibitory substances, decreasing the accumulation of toxic metabolites and phenolic exudation (Weatherhead et al., 1978; Thomas, 2008).

The analysis performed using optical microscopy revealed different stages characteristic for somatic embryogenesis, from early stages of somatic embryos (globular, heart, torpedo) and even cotyledonary (Figure 8 a, b) with two apexes: apical meristem and root.

Histological samples showed no vascular connection with the origin explant or callus (Figure 8c), an aspect characteristic of somatic embryos (Merkle et al., 1995; Yeung, 1995).

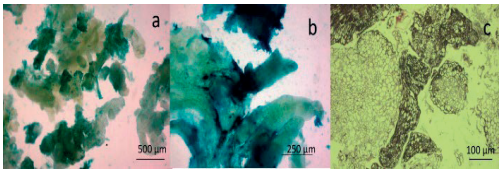

Figure 8. Optical microscopy analysis of embryogenic cultured induced on MSL9 variant with different developmental stages as globular, heart, torpedo, cotiledonary (a, b); c- histological samples revealing somatic embryos structures without vascular connections

The scanning microscopy also confirmed the structures described in optical microscopy (Figure 9).

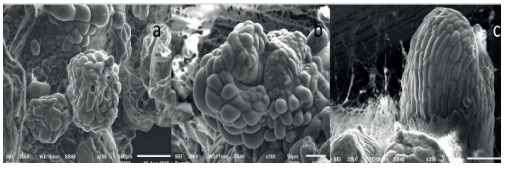

Figure 9. SEM analysis of embryogenic cultures showed somatic embryos (a, b, c)

Early embryogenic globular stage (Figure 9 a, b) and cotyledonary stage (Figure 9 c) were detected. Among different ways of regeneration, somatic embryogenesis is considered to reduce the time necessary for plant propagation, also minimizing genetic changes (Sivanesan & Jeong, 2016).

The culture media variants MSL17-MSL20 led to the formation of a non-regenerative callus, but only variants with auxin dominance and the 2/1 auxin/cytokinin ratio ensured a good growth and survival of the calli. This material can also be used as a supplementary source for secondary metabolites' production through establishment of stables cell lines.

Synthetic seeds or artificial seeds represent a tool with high practical importance, being alginate-encapsulated micropropagules, which can be meristems, apexes or somatic embryos that can behave similarly to classic seeds, being able to develop into plantlets after propagation under *in vitro* or *in vivo* conditions.

Artificial seeds can be used for cryopreservation approaches (Fabre, 1990), such as encapsulation-dehydration or encapsulation-vitrification techniques (Sakai et al., 2008) and represent an efficient strategy for long-term preservation (Kulus, 2016).

After different treatments (dehydration and/or vitrification) and cryostorage, artificial seeds can ensure plant production whenever necessary, based on previously established *in vitro* regeneration protocols for every taxon (Sharma, 2005; Sivanesan & Jeong, 2016).

Besides germplasm preservation, artificial seeds may be used for exchange of plant material between laboratories. Encapsulated material can effectively save many subcultures and eliminate the difficult stage of acclimatization of *in vitro* plants (Qahtan et al., 2019).

Owing to the large-scale production capacity of somatic embryos, this material can easily and successfully be used to prepare synthetic seeds and test different preservation protocols, instead of isolating meristems, a delicate and time consuming procedure.
Testing multiple dehydration procedures as a preliminary step for medium- and long-term preservation, our results showed that immersion for two days in sucrose at 0.25 M and 0.5 M led to an approximately 70% regeneration rate. After a subsequent treatment with 0.75 and 1 M sucrose, the rate decreased to 30%.
Similarly, a sucrose dehydration treatment was effective as a prerequisite for the survival of artificial seeds obtained from embryogenic cultures in *Dianthus nardiformis* exposed to ultralow temperatures (Holobiuc et al., 2023).
Once cultured on an induction medium mainly containing 2.4-D or Dicamba at levels of 0.5-1 mg/L, artificial seeds continued to produce somatic embryos.
In order to develop an appropriate treatment that can ensure plant material survival during ultralow temperatures exposure, further tests need to be performed.

CONCLUSIONS

Over decades, the conservative, qualitative and quantitative aspects of *in vitro* cultures of *L. nivale* have been studied.
Our results revealed that the addition of glutamine and a combination of BAP, kinetin and NAA can be beneficial for stimulating direct morphogenesis starting from aseptic seedling explants.
The growth of plantlets was improved on MS medium added with charcoal 0.5 g/l.
Secondly, our results proved that low levels of 2/4-D or Dicamba successfully induced somatic embryogenesis, further increasing the mass production of this valuable plant, for economic and conservative purposes.
In vitro cultures can efficiently provide biological material necessary for different basic studies without the need to use *ex vitro* plants, for the production of valuable compounds and plant multiplication, with low costs.
The high regeneration rate through somatic embryogenesis can supply a large number of propagules, useful for plant regeneration, for ornamental purpose and also to provide mass production for different important compounds, a process which can undergo all-round the year, independent of environmental factors and in a reduced space.

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