

IN VITRO PROPAGATION AND ENCAPSULATION OF GALATELLA ALBANICA DEGEN SHOOT TIPS

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ABSTRACT. *Galatella albanica* subsp. *paparistoi* Qosja is a threatened endemic species of Albania observed in small subpopulations in the sandy dunes and serpentine substrates. It is assessed as endangered plant, so stabilization of an efficient micropropagation protocol and development of a method for *in vitro* preservation *via* encapsulation of this species is very important. In this investigation, leaf segments were used as explants for tissue culture and various combinations of auxin (NAA) and cytokinin (BAP) were tested as growth regulators for *in vitro* morphogenesis. Among these combinations, 1 mg l⁻¹ NAA + 0.4 mg l⁻¹ BAP (2.5: 1 ratio) resulted optimal for callus induction, meanwhile the addition of 0.4 mg l⁻¹ NAA : 1 mg l⁻¹ BAP (1 : 2.50 ratio) into culture medium was optimal for adventitious shoots regeneration. Regarding multiplication parameters, the highest number of shootlets, and leaves and shootlets height were recorded with 0.1 mg l⁻¹ NAA : 2 mg l⁻¹ BAP containing medium. The *in vitro* shoot tips were encapsulated in alginate beads and dehydrated in sucrose solution in order to develop an artificial seed method. Maximum of percentage of recovery was observed in non-dehydrated encapsulated shoot tips (96.6%), followed by the dehydration in 0.25 M sucrose solution for 1.5 h and then at 0.5 M sucrose solution for 1.5 h (63.3%). The recovered plantlets from the encapsulated shoot tips were normal and grew well on recovery medium. According to the obtained results, it could be concluded that the developed micropropagation and alginate-encapsulation method of *G. albanica* can be applied not only for *in vitro* propagation but also for conservation of its germplasm and collections.

Keywords: *Galatella albanica*, callogenesis, PGRs ratio, micropropagation, encapsulation.

INTRODUCTION

The species *Galatella albanica* Degen (syn. *Aster albanicus*) belongs to the *Asteraceae* family and the floristic elements of the Balkans. As an Albanian endemic species, geographically it can be found in the western part of the country and in serpentine areas of the central and north Albania and the south-west Kosovo. At lower altitudes ca. 10 m, it was observed in the openings of forested sandy dunes in contrary with serpentine areas where it is located from 250 m to 1600 m a.s.l [1, 2, 3, 4]. Despite of the wide distribution range [1, 3, 5] due to the small population size, low number of localities and negative impacts of the heavy metals and the “serpentine syndrome”, the species is assessed as threatened with protection status, EN A1b [6, 7, 8]. Along forested dunes by

coniferous plants, from Durres to Karavasta [5], *G. albanica* subsp. *paparistoi* prefers semi shadow places with warm temperatures of the Mediterranean climate.

In the coniferous forest of Divjaka the Albanian aster is restricted by anthropogenic interference during the uncritical use of this beach area. Although this species grows farther from the beach, towards the forest, it occupies areas near the motorway and therefore risks becoming extinct. The protection measures consist in the conservation of this endemic species through the proclamation of its growing habitats as protected areas or national parks. For these reasons, it is extremely important finding alternative ways for its propagation and conservation.

There are some initial studies of conventional methods of *G. albanica* propagation which conclude that exist some natural germination flaws such as the low germination potentiality, the slow development of roots even that the new leaves of plantlets are well developed. Since many years ago, the *in vitro* propagation method was proposed as the optimal method for producing a large number of plants to move to the natural growth field [9, 10]. In this respect, leaf explant was used for initiation of *in vitro* cultures of *G. albanica* subsp. *paparistoi* [11]. The micropropagation of threatened species using shoots as primary explants was early reported by Lynch and Johnston [12], in case of Georgia Aster (*Symphyotrichum georgianum*). Further more, *in vitro* regeneration and multiplication capacity of *Aster alpinus* L. was studied by Agud et al. [13].

By the other hand, the possibility of plant regeneration and multiplication by tissue culture techniques provides the basis for the establishment of *in vitro* genetic banks for the preservation of plant species of community interest, which are at risk of extinction [14, 15]. The application of *in vitro* methodologies for mass propagation of plants is widespread and ensures a big amount of homogeneity and identical plant material with mother plants which can be used in long term storage (cryopreservation) and mild- or short-term storage without losing viability [16,17]. Recently the new trend of synthetic seeds production offers many advantages in the ability to maintain a high regeneration potentiality after the conservation. The encapsulation (artificial seed) technology is considered a promising approach that can be used for the exchange of plant materials between plant tissue culture laboratories, and also to achieve germplasm conservation and the propagules that are derived from *in vitro* or by micropropagation applied directly in nurseries or in a field [18]. They are considered as a powerful tool for germplasm conservation of elite species with high economic value (cereals, vegetables, fruit plants, ornamentals, aromatic and conifers) and threatened species [19, 20]. Artificial seeds or also called synthetic seeds represent capsules with a gel envelope which contain somatic embryos, axillary and apical buds or stem and root segments [21]. Several gel types are exploited for encapsulation; however, sodium alginate established itself to be the most frequently used matrix because of low cost, gelling properties, and its nontoxic nature [22]. The parameters of encapsulation (mostly alginate coat matrix), dehydration, suitable storage temperature and duration should be optimized for selected species to achieve a high percentage of plant conversion after conservation. The aim of this study is to establish an effective micropropagation protocol using different PGRs ratios and plant recovery from alginate-encapsulated microcuttings of *G. albanica* subsp. *paparistoi* in order to stabilize a primary step for long-term germoplasm conservation process. In our knowledge this is the first report about the synthetic seeds production of this species.

MATERIALS AND METHODS

Explant disinfection and sterilization

Leaf explants of *G. albanica* collected in Divjaka district were used for the stabilization of *in vitro* culture. The explants were firstly washed for 30 min in tap water, treated with 70% (v/v) ethanol for 30 sec. and finally with 0.1% HgCl₂ for 5 min. After each treatment the explants were washed with sterile distilled water.

Callogenesis induction and adventitious shoots regeneration

Leaf explants were inoculated on MS medium [23] supplemented with 0.4 mg l⁻¹ BAP (6-benzylaminopurine) in combination with three concentrations (0.5, 1, and 1.5 mg l⁻¹) of NAA (α -naphthalenacetic acid) for callus formation. After three weeks, the initiated calli were cultured in the regeneration medium containing 0.4 mg l⁻¹ NAA and supplemented with different concentrations (0.5, 1, 1.5 mg l⁻¹) of BAP as shown in Table (1). Data of callus formation frequency (%) and shoots regeneration frequency (%) were recorded.

Table 1. Different combinations of NAA and BAP used for callogenesis, adventitious shoots regeneration and shootlets multiplication of *G. albanica*

Culture stage	NAA (mg l ⁻¹)	BAP (mg l ⁻¹)	NAA: BAP ratio
Callogenesis	0.5	0.4	1.25 : 1
	1	0.4	2.50 : 1
	1.5	0.4	3.75 : 1
adventitious shoots regeneration	0.4	0.5	1 : 1.25
	0.4	1	1 : 2.50
	0.4	1.5	1 : 3.75
Shootlets multiplication	0.1	0.5	1 : 5
	0.1	1	1 : 10
	0.1	2	1 : 20

Shootlets multiplication via subcultures

In this stage, three different concentrations of BAP (0.5, 1, 2 mg l⁻¹) were tested with the addition of 0.1 mg l⁻¹ NAA and 100 mg l⁻¹ of sequestrene 138 6% fully chelated EDDHA as iron source. Morphometric parameters as shoots number, leaves number and shoots length were estimated and compared.

In all cases, the media were supplemented with 3% (w/v) sucrose and 0.7% (w/v) agar. The pH was adjusted at 5.7 before autoclaving at 121°C for 20 min. All the procedure was conducted under laminar flow cabinet conditions and the culture vessels were kept at 25±2°C with a 16 h photoperiod and 2000 lux illumination.

Encapsulation and dehydration

To achieve the optimal polymerization for an effective encapsulation process, two different viscosity (degree of methacrylation) grades i.e., low (10-20 %) and medium (20-40 %) of sodium alginate (Sigma-Aldrich) were tested. Shoot tips (~5 mm) of the plantlets obtained after three subcultures were isolated and then immersed in 3% (w/v) of the two

grades of sodium alginate solution containing MS salts without calcium chloride dihydrate, MS vitamins and 3% (w/v) sucrose. Both explants and sodium alginate solution were then dropped in the 100 mM $\text{CaCl}_2 \times 2\text{H}_2\text{O}$ solution using a sterile 10 ml *Pasteur* pipette in order to achieve “the perfect” bead shape and less contamination. After 30 min needed for $\text{Na}^+/\text{Ca}^{2+}$ ion exchange and beads formation, was followed the dehydration procedure and after that the inoculation in recovery media. Two methods were used for dehydration process and the survival and regeneration percentage was evaluated compared to control:

- a) Immersion for 24 h in 0.25 M sucrose solution;
- b) Immersion for 1.5 h in 0.25 M sucrose solution followed by immersion for 1.5 h at 0.5 M sucrose solution.

Recovery medium

The alginate beads were inoculated under aseptic conditions in the MS nutrient media supplemented with 1 mg l⁻¹ BAP (6-benzylaminopurine) and 0.1 mg l⁻¹ NAA (α -naphthalene acetic acid), 3% (w/v) sucrose, 0.7% (w/v) agar and grown in the growth chamber.

The control group included explants that were placed for recovery without undergoing the dehydration process.

Growth chamber conditions

The cultures of the proliferation stage were grown in the growth chamber at temperature of $25^\circ \pm 2^\circ \text{C}$ in a 16 h/8 h light/dark regime with cool, white fluorescent light of intensity $43.4 \mu\text{mol m}^{-2} \text{s}^{-1}$.

Statistical analysis

All experiments are repeated at least three times. Experimental data was elaborated by Tukey-Kramer, Student's methods and the analysis of variance (ANOVA) with JMP 7.0 statistical software.

RESULTS AND DISCUSSION

Callogenesis and adventitious shoots regeneration

For callus induction from leaf explants of *Galatella albanica* Degen, three different combinations of NAA and BAP were tested. After 3 - 4 days from inoculation the uniformity of leaf explants was disrupted, especially at the edges of leaf sections. It appears that the callus formed is derived from basal cells which are in contact with the nutrient medium, as a response to both endogenous and exogenous hormones. Callus was observed 3 weeks after inoculation for the most part of the explants (Fig. 1a). Both cell division and callus production require additions of NNA auxin and cytokinin BAP at a given ratio. The obtained results showed that PGRs ratio is the most important for callus induction rate (Fig. 2a). Among the tested growth regulators, the combination of 1 mg l⁻¹ NAA + 0.4 mg l⁻¹ BAP (2.5: 1 ratio) was the most effective in which 71% of the explants gave friable callus. While, the two other combinations 0.5 mg l⁻¹ NAA + 0.4 mg l⁻¹ and 1.5 mg l⁻¹ NAA + 0.4 mg l⁻¹ (1.25 : 1 and 3.75 : 1 ratios) didn't resulted as much effective and the explants showed a callus induction rate specifically 55 and 60% respectively. For

adventitious shoots regeneration the obtained calli were transferred in three different nutrient media where the NAA : BAP were the opposite with those tested in the first stage of callogenesis. In all cases compact callus was developed and shoots regeneration was observed (Fig. 1b). Even in this case, PGRs ratio affected shoot regeneration frequency. Herein, the 0.4 mg l⁻¹ NAA : 1 mg l⁻¹ BAP (1 : 2.50 ratio) resulted the most effective combination for adventitious shoot formation (65 %), in comparison with the other concentrations (0.5 and 1.5 mg l⁻¹), 50 and 30% respectively (Fig. 2b).

The most predominant element that eventually regulated the frequency of callogenesis in plant tissues is the type of PGRs and their concentrations. Quite a few auxin and cytokinin sources were used, either alone or in combinations to ensure effective morphogenetic response. The results of the present experiment demonstrated that callus induction frequencies from leaf explant of *G. albanica* is strongly affected by combination of NAA and BAP added to cultures medium. Out of different combinations, 1 mg l⁻¹ NAA + 0.4 mg l⁻¹ BAP was found optimum for callus induction response and the observed callus was light green in color with soft texture. Calli raised from leaf explant produced high frequency of adventitious shoot using MS medium contained 0.4 mg l⁻¹ NAA : 1 mg l⁻¹ BAP. The results are in accordance with those reported by Qendro et al. [8] in their study on *G. albanica* subsp. *paparistoi* Qosja. They found that MS culture medium with the combination of cytokinin BAP and auxin NAA appears to be optimal for the start of callusogenesis. Similarly, in their study on *Aster caucasicus* and *Aster sedifolius* using different auxins : cytokinins ratio, Minutolo et al. [24] found that the 2 : 1 ratio of 2.4-D : BAP was the best level for callus mass formation. From other reports of callogenesis induction on Albanian threatened species [25, 26] or reports on species of interest to Salento flora, Italy [27] it turns out that among different variants of combinations of the auxins (NAA and IAA) with cytokinins (BAP, kinetin), the optimal combination is 1.25 : 1 BAP : NAA ratio. On the other hand, high regeneration rate of *Aster alpinus* L. were obtained using MS medium supplemented with low concentrations of growth regulators (0.4 mg/l BA, K, 2iP and 0.2 mg/l NAA) [13]. In this respect, Boo et al. [28] examined the callus induction and embryogenic capacities of three explants, including leaves, petioles, and roots of *Aster scaber*, on media containing different combinations of NAA and BA. The optimum concentrations of NAA and BA for the production of embryogenic calli were 5.0 µM and 0.05 µM, respectively. Media containing higher concentrations of auxin and cytokinin (such as 25 µM NAA and 25 µM BA) were suitable for shoot regeneration, especially for leaf-derived calli.

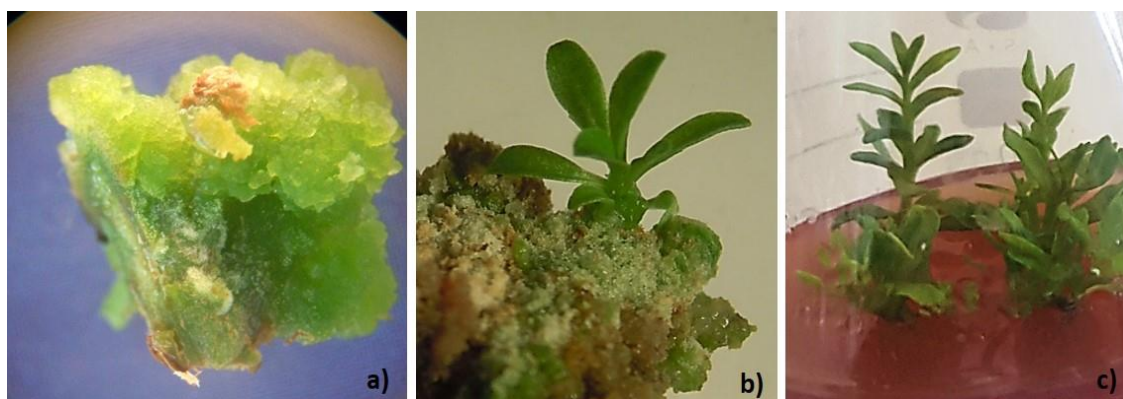


Fig. 1. (a) Callus induction, (b) shoot regeneration and (c) shootlets multiplication of *Galatella albanica* seed germination

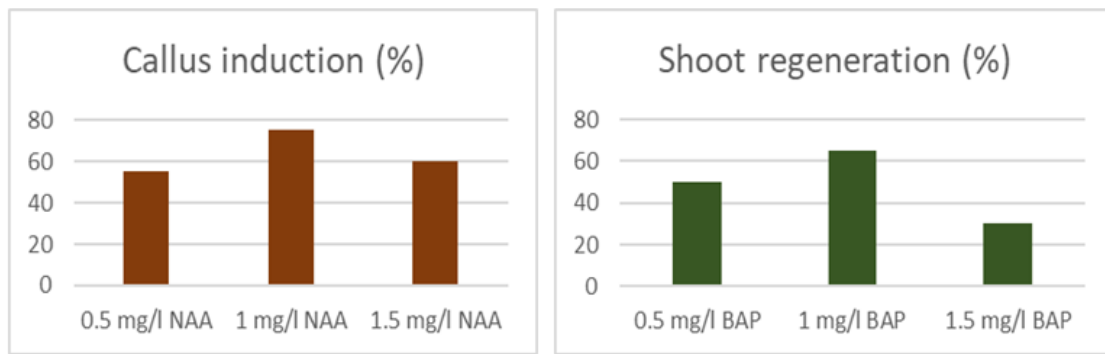


Fig. 2. (a) Callus induction and (b) shoot regeneration rates in specific combinations of BAP:NAA

Shootlets multiplication

Both auxin and cytokinin are usually added to tissue culture media in order to obtain morphogenesis. In combination with auxins, cytokinins stimulate cell division even in non-meristematic tissues. Furthermore, the ratio of cytokinins to auxins controls cell differentiation and when the ratio is in the favor of cytokinins, shoot formation takes place. In the present investigation, adventitious shoots obtained from the previous stage were inoculated in multiplication media containing three concentrations (0.5, 1 and 2 mg l⁻¹) of BAP combined with 1 mg l⁻¹ of NAA. The morphogenetic parameters presented as shootlets and leaves numbers and shootlet height were evaluated. After 5 days of culture development new axillary shoots formation was observed due to the high concentration of BAP in combination with NAA (Fig. 1c). The highest number of shootlets, leaves and shootlets highest was recorded with 0.1 mg l⁻¹ NAA : 2 mg l⁻¹ BAP (1 : 20 ratio) (Fig. 3). For this stage of micropropagation, mean values of the evaluated biometric parameters show a significant difference among them. As shown by the results, increasing BAP concentration in the nutrient media rapidly increases biometric parameters, leading in large quantity of shootlets formation. Thus, it is clear that a specific ratio NAA : BAP is important for obtaining large number of plantlets. The results also show that the combination of high levels of BAP is much better for shootlets proliferation of *G. albanica* than the combination of low levels. The effect of cytokinins on increasing the number of shoots during micropropagation of *Compositae* plants was reported by other authors [29, 30]. Furthermore, in their study on micropropagation of *Inula germanica* L (Asteraceae), Trejgell et al. [31] evidenced the 1 : 10 ratio between NAA and BAP was an optimal one for multiple shoot formation through direct organogenesis. However, in micropropagation of Georgia Aster, *Symphotrichum georgianum* (Alexander) Nesom, Lynch and Johnston [12] mentioned that shoot multiplication and elongation occurred on half-strength MS salts containing 0.1 mg/L BA and 0.2 mg/L GA3.

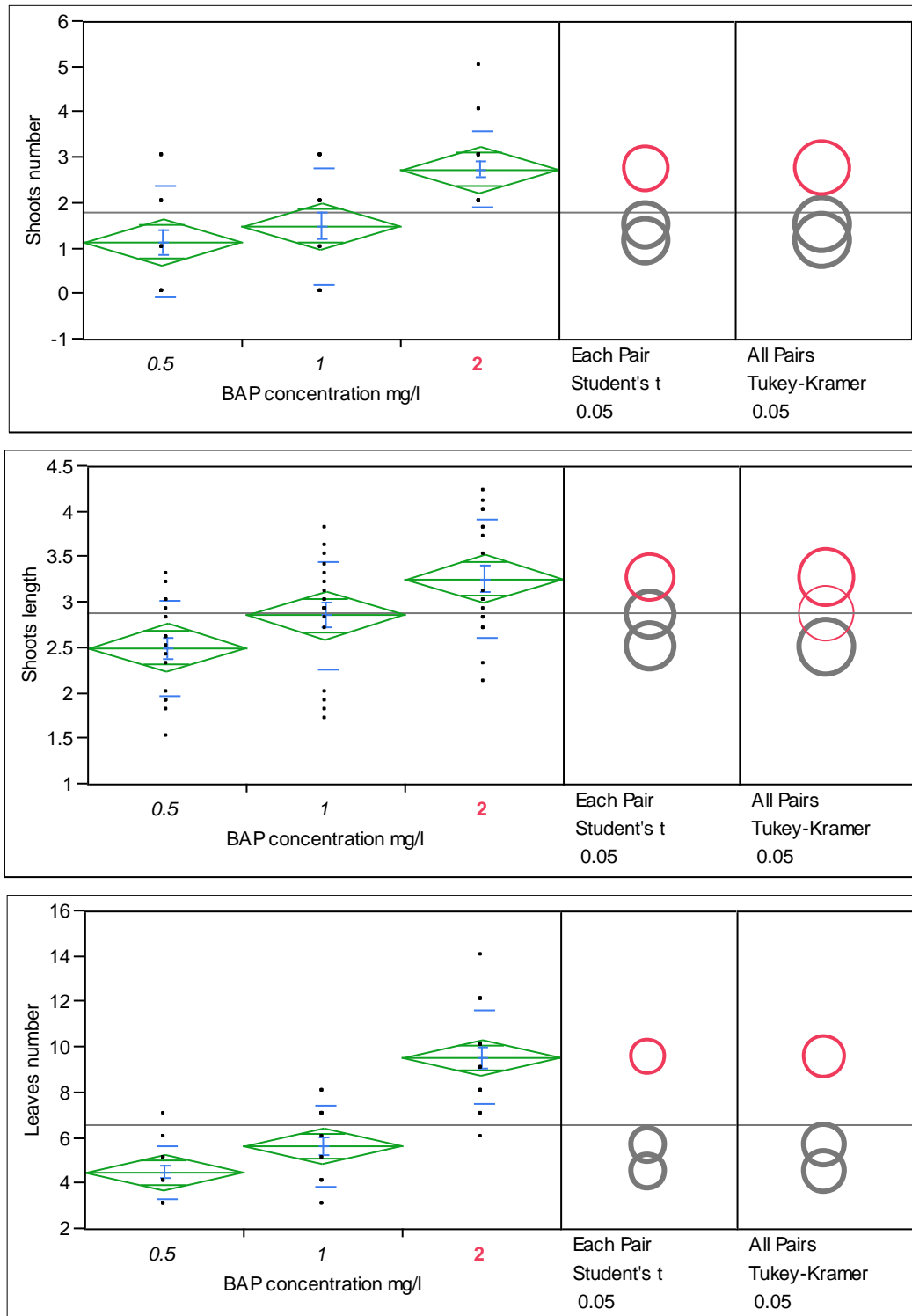


Fig. 3. The effect of BAP concentration on shootlets number, shootlets length and leaves number of *Galatella albanica* grown in vitro

Encapsulation of explants and plantlets recovery

For this study, shoot tips derived from *in vitro* grown shootlets were used as explant for synthetic seeds production. For the encapsulation, two viscosity grades (low and medium) of sodium alginate were used for beads formation. The beads formed by low

viscosity sodium alginate were almost transparent in colour, fragile and not rounded (Fig. 4a). By the other hand, the medium viscosity of sodium alginate salt gave better beads in shape, resistance and handling (Fig. 4b). This may be due to the fact that low viscosity sodium alginate has a higher flow rate than medium one.

After two dehydration procedures, survival and regeneration percentage of encapsulated shoot tips were calculated and compared with the non-dehydrated beads which used as control. One week after the inoculation in the recovering nutrient medium (MS supplemented with 1 mg l^{-1} BAP and 0.1 mg l^{-1} NAA), the encapsulated shoot tips started to get bigger and to break the alginate capsule (Fig. 4c). Then, the encapsulated shoot tips showed the emergence of weak shootlets (Fig. 4d). After four weeks, the shootlets well developed on the recovering medium (Fig. 4e). From the obtained results, it was observed that all of beads that were inoculated without dehydration survived and 96% of them regenerated plantlets (Fig. 5). The recovered plantlets from this treatment were robust and had good vegetative growth. Dehydration for 24 hours in 0.25M sucrose solution resulted non effective because the major part of the explants didn't survive. However, the beads dehydrated with 0.25M sucrose solution followed by 0.5 M one (1.5 hour each treatment) survived at 100% but showed a lower regrowth percentage (66.1%) compared with control. Interestingly, it was noticed a spontaneous flowering in the control plantlets, where 22 % of them gave flowers one month after fully recovering (Fig. 4f). This phenomenon was not observed in the dehydrated explants. Transition from the vegetative to the reproductive (flowering) phase was poorly understood, but may be due to the physical factor caused by encapsulation without dehydration.

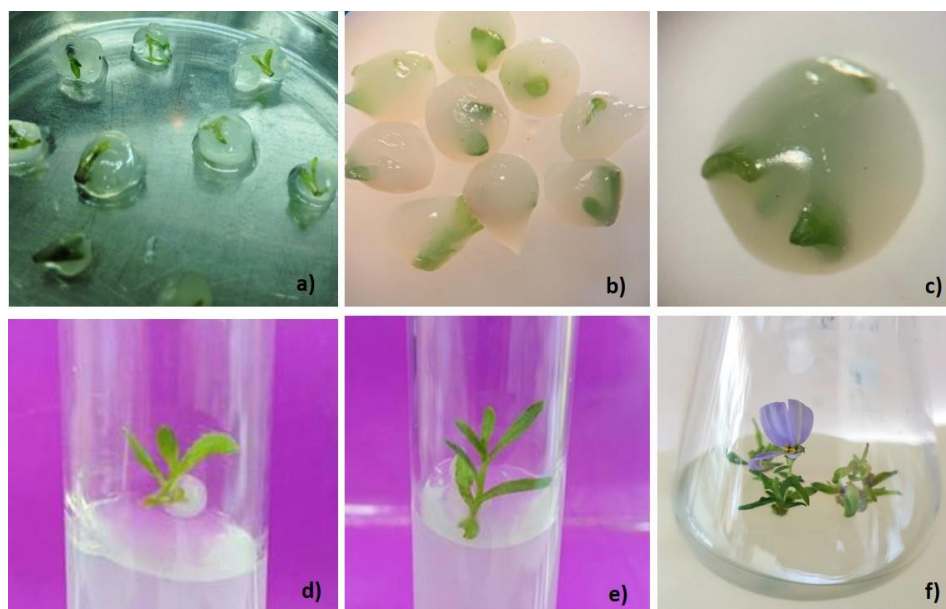


Fig. 4. (a) Beads formation in low viscosity sodium alginate solution (b) Beads formation in medium viscosity sodium alginate solution, (c) Emergence of shootlets from sodium alginate capsule, (d) Shootlets development two weeks after the inoculation in the recovery medium, (e); Complete development of plantlets and (f) In vitro flowering of plantlets derived from non-dehydrated capsules.

The artificial seeds technology based on vegetative micropropagules may be useful not only in large-scale propagation but also in short- and long-term conservation and germplasm exchange of desirable species genotypes. Choosing shoot tip explants for artificial seeds production leads to true clones and ensure the genetic stability. In this respect, there are so many successful reports about shoot tips encapsulation of different plants such as banana [32], woody species [33], etc. For this purpose, alginate hydrogel is used as a matrix for the encapsulation of shoot tips. It is frequently used for this reason due to its moderate viscosity and low spinnability of solution, low toxicity for encapsulated explants as well as quick jellification, low cost and biocompatibility characteristics [34]. The major principle involved in this process is based on the ion exchange between Na^+ in sodium alginate and Ca^{2+} in calcium chloride which results in round and firm beads formation [35]. As it has been reported, the degree of viscosity of sodium alginate used, CaCl_2 concentration and curing time are important parameters to determine the permeability, resistance and hardness of beads [36]. All encapsulated shoot tips of *Galatella albanica* assessed in the present study showed regrowth with high percentages and satisfactory development time. Dehydration of shoot tips induced by sucrose during encapsulation had slight negative effects on both survival and recovering percentages since the best percentages of survival and regrowth were observed with no dehydration treatment. The finding of our study is supported by the results of Sandoval-Yugar et al. [37]. They found that the microshoots of banan encapsulated in sucrose-free capsules had a high potential to convert into plantlets on culture medium. Similar, Mubbarakh et al. [38] in their study on *Rosa hybrida* L. cv. Helmut Schmidt stated that when the beads were dehydrated for more than 3 hours there was a decrease in viability. However, Katouzi et al. [16] reported that artificial seed matrix of sunflower should be supplemented with sucrose which increases the tolerance to dehydration and maintains the tissue viability during long-term conservation in liquid nitrogen (-196°C).

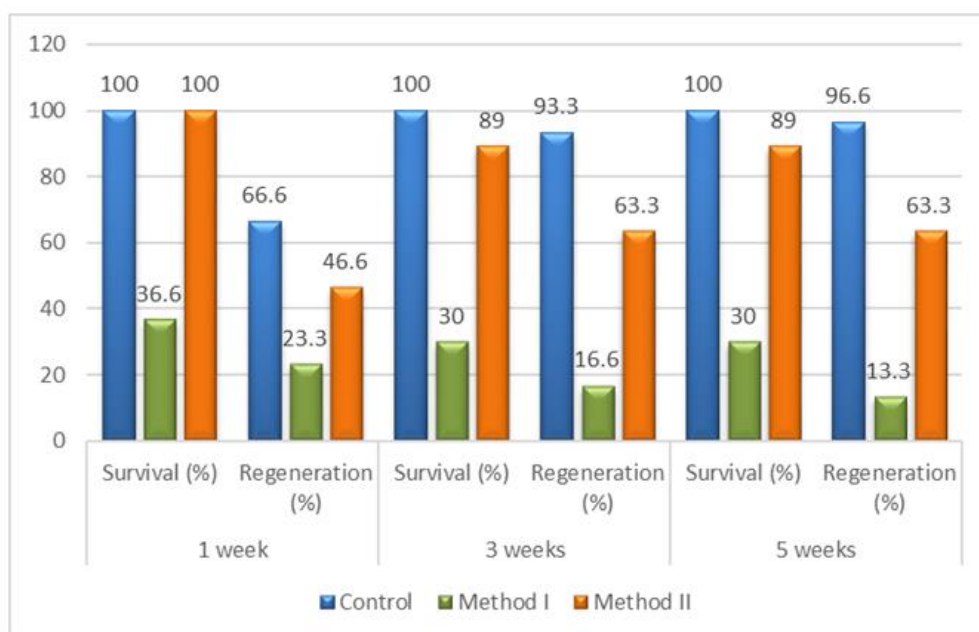


Fig. 5. Survival and regeneration of encapsulated shoot tips of *G. albanica* dehydrated by immersion for 24 h in 0.25 M sucrose (Method I), immersion for 1.5 h in 0.25 M sucrose followed by 1.5 h in 0.5 M sucrose (II) and non-dehydrated (control).

CONCLUSION

Micropropagation and encapsulation of shoot tips of *Galatella albanica* can offer an efficient technique for its clonal propagation and also enables the establishment of basal collection for *in vitro* germplasm conservation. On the basis of the obtained results in this study, it can be concluded that it is important to stabilize a proper NAA – BAP ratio for enhancing callogenesis and multiplication of *G. albanica* species. For callogenesis induction and adventive shoot regeneration it was observed an opposite ratio of NAA : BAP, specifically 2.50 : 1 and 1 : 2.50. Meanwhile, regarding multiplication, addition of BAP in the nutrient media at 2 mg l⁻¹ and NAA at 0.1 mg l⁻¹ resulted as the most effective combination. Another important conclusion is the effective plant recovery from alginate-encapsulation microcuttings. Dehydration for more than 3 hours in sucrose solution leads to low regeneration percentage. The results proved that the protocols of micropropagation and recovering from alginate-encapsulate microcuttings are well established and might be very useful for long-term conservation and genetic collections establishment of *G. albanica*.

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