In vitro Preservation by Encapsulation of Shoot Tips of *Aerva lanata* (L.) Juss. ex Schult. as a Rare Medicinal Plant

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***Abstract* – *Aerva lanata* (Linn) belongs to the family Amaranthaceae is a rare perennial shrub or erect herb growing in the Gabal Elba protected southeast corner of the Eastern Desert of Egypt. In order to increase the demand for medicinal wild plants in the drug industry, care must be taken to the necessity of preserving those plants, especially rare ones. An efficient method of in vitro propagation, synthetic seed production protocol was established for the conservation of *A. lanata*. Shoot tips excised from in vitro the proliferated shoots were encapsulated in calcium alginate beads. A gelling matrix of 3% sodium alginate and 100mM calcium chloride was found the most suitable for formation of ideal calcium alginate beads. Shoot tips have been preserved in calcium alginate beads containing calcium alginate for five months. Three matrices; water, medium of Murashige and Skoog and medium of Murashige and Skoog containing 3% sucrose and measured for storage at 24 ± 2 and 4C°. The regeneration efficiency of *A. lanata* shoot tips encapsulated was investigated. On the Murashige and Skoog medium, 100% viability and high regrowth percentage and productivity of recovered shoot tips were observed at 4°C in all examined alginate matrices. Murashige and Skoog medium with sucrose, however, were considered superior. Rooting was carried out under the same medium and 73.3% of regenerated plantlets under greenhouse conditions were effectively hardened. The regeneration ability and regrowth of encapsulated shoot tips during storage are decreased on a monthly basis. The derived protocol can be used successfully to retain encapsulated *A. lanata* shoot tips for five months at 4°C without any growth regulator–treatments. The encapsulation technique could also be used as an alternative method of propagation and support the conservation of the desired elite genotype of *A. lanata* by cost-effect germplasm.**

***Keywords* – Conservation, Germplasm Exchange, Short-term Storage, Synthetic Seeds.**

I. INTRODUCTION

Encapsulation is considered one of the important means of preserving and propagating on a large scale, preserving germplasm and safe transportation between laboratories and the country. Moreover, it is effective in short term conservation and low in production costs while ensuring that no genetic changes occur *in vitro* the plant material [1, 2]. In addition, It is an effective and promising method for reproduction and conservation of premium species, unconventional seeds, and rare medicinal wild plants [3]. Specifically, the species somatic embryogensis is unachieved. Recently, there has been a growing interest in the use of synthetic seeds developed by encapsulation processing, which is quite an impeccable path for safer protection and species exchange. Option genotypes and sterile unstable genotypes have opened a new path with tremendous potential for the preservation and large-scale micropropagation of exclusive uncommon hybrids. The attention of researchers has lately been centred on encapsulation technologies for germplasm distribution and other various analytical studies [4]. However, the primary account of the production of artificial seeds was reported by Kitto and Janic [5], and Murashige noted the notion of the same [6]. By introducing encapsulated shoot tip and nodal section, rare and endangered medicinal plants can be protected from numerous adversities. Significant attention has been given to implementing encapsulated shoot tip in clonal propagation and short-term storage to get an advantage

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on all these problems [7]. Synthetic seeds are produced by using encapsulation technology that are widely used to conserve and commercialize economically important medicinal plants [8]. By producing synthetic seeds, significant progress has been made for a wild range of plant species [9]. The plant's survival is constantly threatened by overgrazing and random selection of wild medicinal collection plants, which has resulted in a deficiency of these plants and a threatened with extinction restricted range. Therefore, new and practical methods must be found in order to preserve and multiply these plants. Therefore, an appropriate technique of macro- or micropropagation needs desperately to be developed to recover the wild population depleting and preserve the species' genetic resources [10]. In order to increase the demand for medicinal wild plants in the drug industry, care must be taken to the necessity of preserving those plants, especially rare ones. It is known that ex situ conservation seeds in gene banks under low temperatures is the most widespread way. However, this method of preserving the seeds may face many difficulties, including the loss of seed viability with the long storage period. It is worth noting that the conservation of wild plants, using tissue culture technique (synthetic seeds) and using different explants such as shoot tips, stem segments play an active and vital role in preserving wild plant species, especially rare and endangered plants. *Aerva lanata* from the family Amaranthaceae is a rare perennial shrub or erect herb growing in the Gabal Elba protected southeast corner of the Eastern Desert of Egypt [11] the plant has effective secondary compounds that are used in curative applications in traditional and folk medicine in many regions of the world [12]. These active compounds consist of phytochemicals such as flavonoids, alkaloids, phenolics, steroids, terpenoids, tannin and saponins [13, 14, 15]. These compounds are known for their properties, pharmacological activity and use widely such as antiurolithiatic, diuretic, hepatoprotective, anticancer, antimicrobial, antioxidant, antihyper lipidemic, antidiabetic, immunomodulatory, antihelminthic and antiflammatory [14, 16, 17]. There are other reports available where low temperature (4C°) was suggested for short-term storage [18, 19]. Synthetic seeds are used to preserve many endangered medicinal plants because they are small in size, easy to handle, low cost and exchange germplasm. It also allows for the exchange of free pathogen propagules across international borders, as well as the avoidance of bulk plant transportation, quarantine, and disease outbreaks. Where the ability to convert plants is preserved even after storage under room conditions or in liquid nitrogen [20, 21]. As far as the researcher knows, there is no study on preserving the *A. lanata* by Encapsulation shoot tips. In *Aerva lanata*, synthetic seeds were produced to provide efficient storage, germplasm exchange and distribution of synthetic seed production, encapsulating the shoot tips. Hence, this study aimed to develop an efficient encapsulation shoot tips for conserving *A. lanata* as a rare medicinal herb in Egypt.

II. Material and Methods

Plant Material:

Stem segments of young and healthy branches of *Aerva lanata* were collected from plants grown in the Gebel Elba protected, southeast corner of the Eastern Desert of Egypt. Stem segments were cultured in Murashige and Skoog (MS) medium [22] containing 30 g/l sucrose and 0.1 mg/l myo-inosetol supplemented with different concentrations of benzyladenin (BA) (0.0, 2.22, 4.44, 8.90 and 17.80 µM) (Duchefa, Haarlem, the Netherlands) for initiation and multiplication stage. Subculture were every 4 weeks intervals. The PH of the medium was adjusted to 5.7 to prior 5.8 to adding 2.75 g/l phytagel (Duchefa, Haarlem, the Netherlands). The medium was dispensed in large jars (350 ml) containing 40 m and autoclaved at 121C° at a pressure of 1.1 kg/ cm^2 for 20

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minutes (Harvey sterilemax autoclave, Thermo Scinentific, USA). All cultures were incubated at a temperature of 25 ± 2°C and photoperiod for 16 hours with light intensity of 20 M Mol/m_2/ s (F140 tgd/ 38, Toshiba) and under relative humidity of 60-70 %.

Preparation of Encapsulation Matrixes:

Three kinds of alginate matrixes were used in this experiment. To prepare each, sodium alginate solution 3% (w/v) (Na-alginate; CDH, India) was added to 100 mM distilled water (M_1), 100 mm full strength MS (M_2) and 100 mm full strength MS medium with 3% sucrose (M_3). The polymerization solution was prepared with 100 mm calcium chloride (CaC_2 - $2H_2O$; Merck, Germany). All solutions were autoclaved for 20 min. at a pressure of 1.1 kg cm^2 and a temperature of 121°C after adjusting the PH to 5.7 to 5.8.

Encapsulation:

For encapsulation, 2.5-3 mm shoot tips were isolated from an *in vitro* stock culture of *Aerva lanata.* Shoot tips were put into 3% sodium alginate solutions, then by using autoclavable micropipette with sterile plastic tips they were dropped into $CaCl_2$. 2 H_2O solution and was left form 30 min for proper polymerization. After harding, the capsules were washed with distilled water to remove the traces of calcium chloride. (Photo: 1 A and A).

Encapsulation Storage:

The capsules were placed on filter paper in order to dry them. After wards, encapsulated shoot tips of each alginate matrix were stored in petri dishes containing water agar medium, which were parafilmed to avoide contamination and placed in dark at two temperatures 4°C and 25° ± 2 for five months. Non- capsulated shoot tips were used as a control. Recovery or regeneration evaluation of encapsulated shoot tips. After each storage period, encapsulated and non-encapsulated shoot tips were cultured on MS medium supplemented with 1 mg/l BA+ 0.4 mg/l NAA (the best multiplication medium). In addition to direct culturing after capsulation, it is considered zero storage. All cultures were maintained, as previously described, for 4 weeks. Percentage of viability, regrowth, mean number and length (cm) of shoots per explant and rooting percentage were recorded for 5 months. For rooting, separated the plantlets and roots washed with sterile distilled water to get rid of medium. Then, the plantlets were transferred to pots containing garden soil mixed with sand and peat moss at a 1:1:1 ratio. The pots were covered with polyethylene bags to mointain high relative humidity and kept in green-house. After two weeks, polyethylene bags were removed gradually. The plantlets were irrigated with half strength medium every four days.

Data Analysis:

All treatments were conducted with 10 replicates and repeated three times. The data was analyzed using a NOVA program for statistical analysis. Data means of each observation were compared using Duncan's multiple range test at 5% [23] at $P \leq 0.05$.

III. Results and Discussion

Effect of Storage Temperature and Alginate Matrix on the Regeneration of Encapsulated Shoot Tips

Alginate encapsulation of shoot tips along with preservation offers a strong possibility for germplasme stora-

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-ge and plant regeneration [24, 25]. In general, shoot tips record-superior growth characteristics [26, 27] and they are the most frequently used unipolar propagules for clonal propagation [28]. From the pervious studies, shoot tip for synthetic seeds preparation were the most responsive because of the greater mitotic activity in the meristem [29, 30]. Storge of synthetic seeds at lower temperature was recorded successful for vegetative propagules of many medicinal plant [31]. In this study, two storage temperatures of 4 and 25 ± 2 C° were used to determine the best one for storage. From the observations, the encapsulation deposited at 24 ± 2°C sprouted over 10 days and it became clear that the low temperature at 4C° was the ideal temperature above 25 ± 2 C°, and that the temperature at 25C° was not appropriate and insufficient. The most suitable storage for synthetic seeds with corresponding high regrowth of some medicinal plants was determined to be the optimum minimum temperature for short medium terms at 4C° [32, 33, 34]. In terms of their regrowth potential, it seems like cold storage synthetic seeds are superior to those stored in room for all the growth parameters tested. A promising 4C° degree of storage for regrowth percentage as well as shoot and root production was observed [35]. Changes in metabolic content under the control of low temperatures may be associated with (a) the catalytic activity or stabilization of enzymes involved in biosynthesis or degradation of particular compounds; (b) the biosynthesis of compounds induced by cellular damage; (c) the regulation of the concentration of the compounds involved in homeostasis maintenance; or (d). Biosynthesis and aggregation of compounds that are active in tolerance growth [36]. In addition, lower temperature allows slower desiccation, which demonstrates optimum storage conditions for *Aerva lanata* synthetic seed on a short-term basis. A critical factor in plant regeneration after storage is the structure of the alginate matrix used for encapsulation. The optimization of the alginate matrix structure improves the regeneration potential of the encapsulated shoot tips and the procedure will thus be more effective and applicable. Non-encapsulated and encapsulated of *A. lanata* shoot tip within one week after moving them to the regeneration medium, shoot formation was seen in 3% Na-alginate and 100 mM CaCl2.2H2O. After five months of preservation, all evaluated Na-alginate matrix compositions demonstrated 100% viability of encapsulated shoot tips (Table 1). However, the percentage of regrowth and the number and length of regenerated shoots were lower in encapsulated shoot tips compared to none capsulated because the non-encapsulated shoot tips are directly in contact with the nutrient medium (photo1B). With respect to regrowth percentage and shoot number and length, the Na-alginate matrix containing MS medium displayed significant improvement over that containing distilled water **(Photo 1C)**. This treatment resulted in an 83.33 % regrowth percentage of encapsulated shoot tips, forming 7.33 shoots per explant with a length of 3.37 cm, after five months of storage **(Photo 1D)**. This result demonstrates that the main ingredients of the Na-alginate matrix are MS nutrients for regrowth and regeneration. A significant factor for the successful technique of growing artificial seeds is the addition of nutrients to the encapsulated, increasing the germination capacity and viability of these seeds [37]. These components are known as artificial endosperm and play a major role in artificial seed storage [38].

Table 1. Effect of sodium alginate matrix composition on the survival, regrowth percentage and growth parameters of encapsulated shoot tip of *Aerva lanata* after five months.

Alginate Matrix Composition	**Survival %**	**Regrowth %**	**Number of Shoots/Bead**	**Mean Shoot Length (cm)**	**Rooting %**
Shoot tips (non-encapsulated)	100	100a	11.52a	6.3a	73.3
Alginate	100	76.66d	6.43d	1.2d	65.4

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Alginate Matrix Composition	Survival %	Regrowth %	Number of Shoots/Bead	Mean Shoot Length (cm)	Rooting %
Alginate + MS medium	100	83.33c	7.33c	3.37c	69.3
Alginate + MS + 3% Sucrose	100	90.00b	10.12b	4.3b	70.1

Means followed by the same letter within a column are not significantly different at $P \leq 0.05$.

Also, the encapsulated can serve as a source of nutrients that can increase the explant's survival and speed up its growth [39, 40]. Sucrose is known to provide an *in vitro* supply of carbon and improved plant recovery through its shoot tip encapsulated in the alginate matrix. When sucrose was supplemented in the nutrient medium, the regrowth percentage and shoot number and length gave their optimum values **(Photo 1D)**. After five months of preservation, 90% of encapsulated shoot tips regrew 10.1 shoots per explant with a mean length of 4.3 cm using the MS medium containing 3% sucrose for encapsulation, possibly through its nutritional ability. The source of carbon has been used as energy to encourage tissue regeneration [41]. Sucrose is an essential ingredient of the sodium alginate matrix, sucrose is the most common carbohydrate used in the *in vitro* culture of plants as a carbon and energy source [42]. However, a matrix containing alginates, as stated by Kaviani [43]. Sucrose is key to encapsulation cryogenic preservation because sucrose improves dehydration resistance and positively influences the viability of the tissues. The significant deficiency of sucrose in the alginate matrix of encapsulated shoot tips contributes to the lack of shoot tip access and re-growth [44]. It has been shown that the addition of sucrose to the encapsulated alginate matrix increases efficiency in explant development and re-growth [45].

Effect of Storage Duration on Regeneration of Encapsulated Shoot Tips

The ability of the encapsulated shoot tip to retain their viability and regeneration after storage was one of the most important success factors in this technique. Humidity and low temperatures were necessary conditions for viability retention and thus for the storage of encapsulated shoot tips. The reaction to the regeneration of encapsulated shoot tips was slightly impacted at 4C°, up to five months, by increasing storage duration.

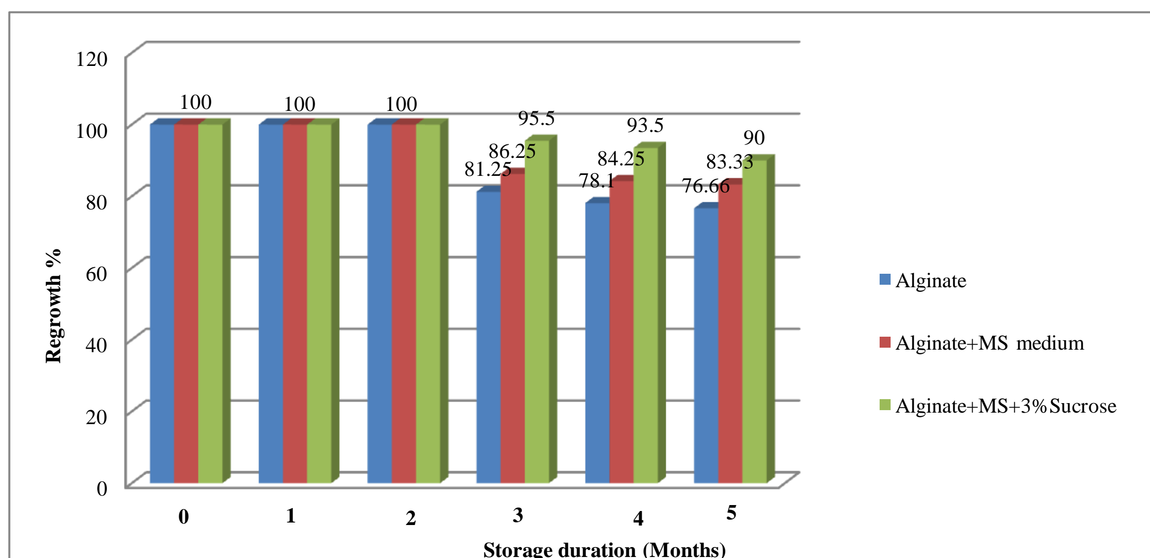

Fig. 1. Effect of different storage duration on regrowth percentage of encapsulated shoot tips of *Aerva lanata* in Na-alginate of different matries, after four weeks from culturing.

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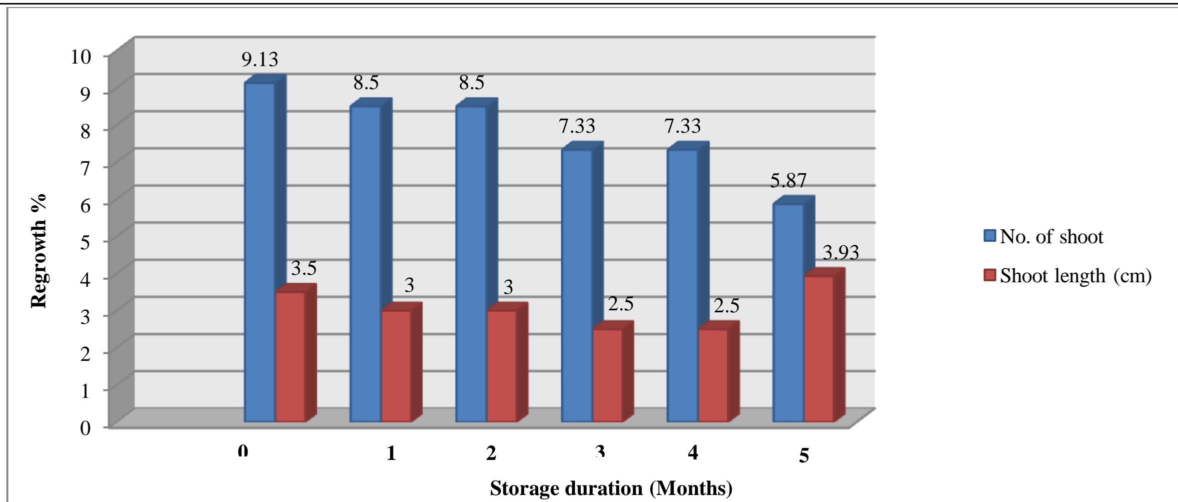

Fig. 2. Effect ofdifferent storage duration on regrowth performance of encapsulated shoot tips of *Aerva lanata* in Na-alginate matrix containing water after four weeks from culturing.

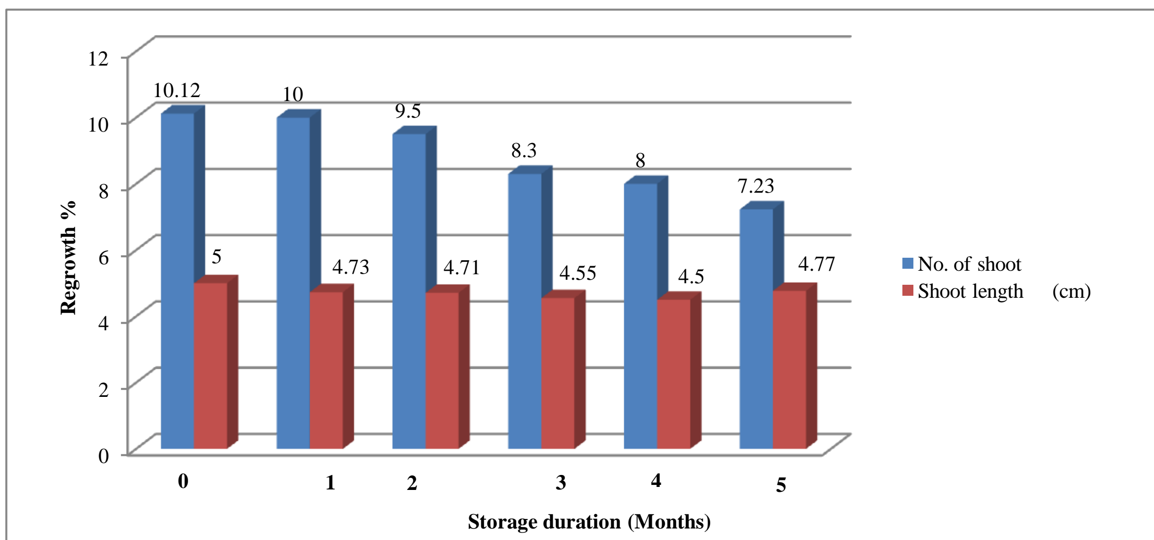

Fig. 3. Effect of different storage duration on regrowth performance of encapsulated shoot tips of *Aerva lanata* in Na-alginate matrix containing MS medium after four weeks from culturing.

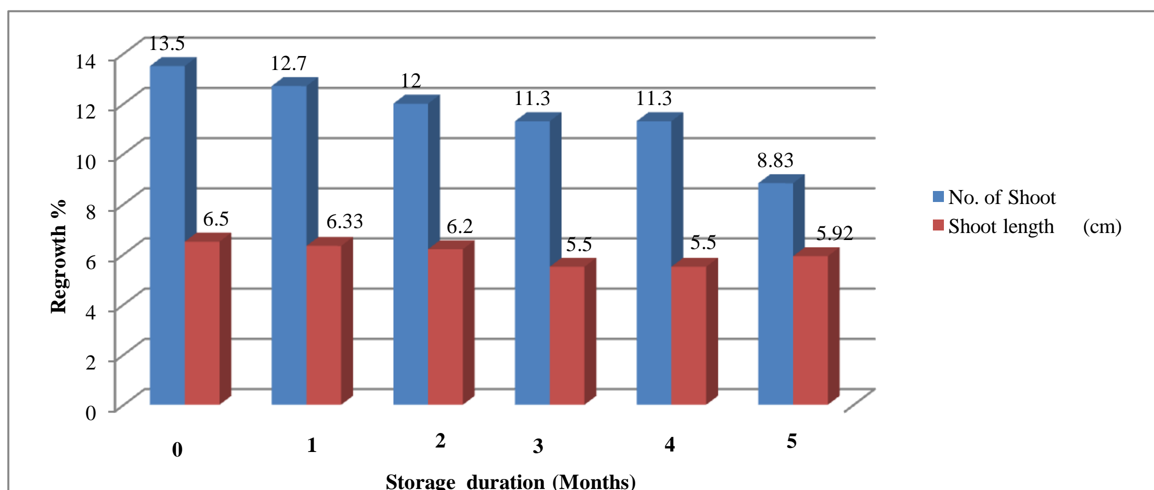

Fig. 4. Effect of different storage duration on regrowth parameters of encapsulated shoot tip of *Aerva lanata* in sodium alginate matrix containing MS medium with 3% sucrose after four weeks from culturing.

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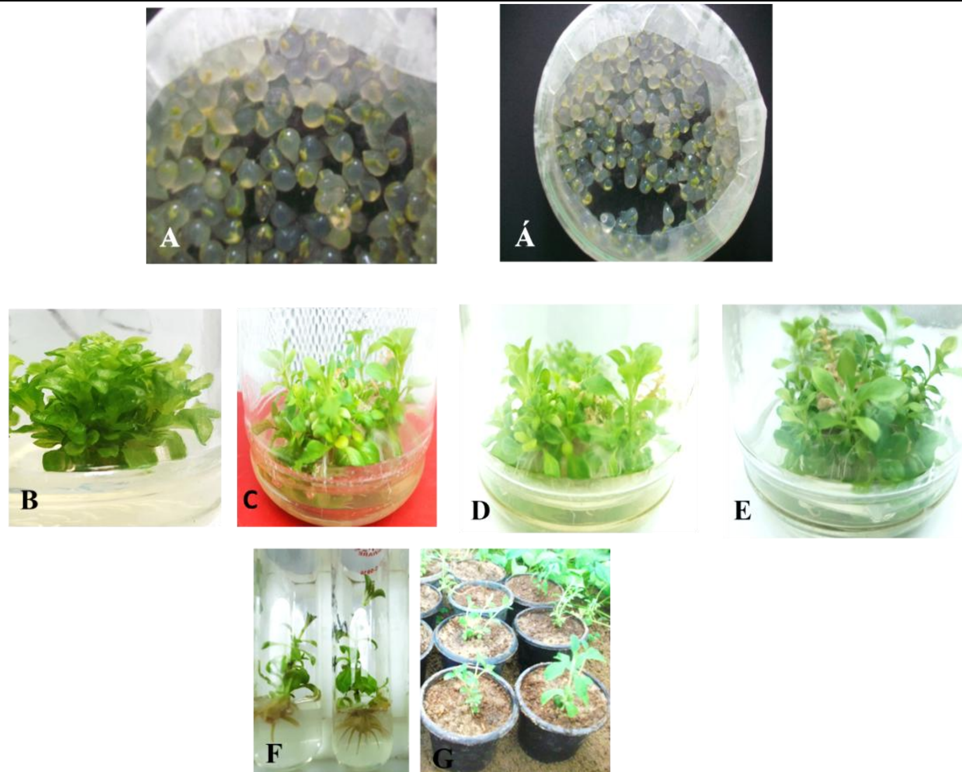

Photo 1. Plantlets regeneration of *A. lanata* from encapsulated shoot tips. A., A Shoot tips encapsulated in calcium alginate beads, B. non-capsulated shoot tips, C. encapsulated shoot tips in distilled water, D. encapsulated shoot tips in MS medium, encapsulated shoot tips in MS medium plus 3% sucrose , F. plantlets with well developed shoots and roots, G. well-hardened plants in plastic pots after three weeks from transfer to greenhouse following five months of storage at 4 C°.

The percentage of regrowth and the mean number and length of shoots produced from the encapsulated shoot tips decreased during the storage period for each tested Na-alginate matrix composition following storage at 4 C. (Figure 1-4). From the previous results, it was observed that reduction in plant recovery from stored encapsulated shoot tips may be attributable to inhibited alginate matrix respiration of the tissues or a depletion of moisture due to partial desiccation found during storage [46, 34]. Also, in Daucus carota, Bazinet et al., [47] reported that the regeneration rate of plants after storage was limited by lack of viability caused by mechanical restrictions or diffusion limitations.

A successful preservation system is measured by the success of plants outside the protocol. This is a critical issue for the preservation system. Well-developed rooted plantlets regenerated from encapsulated shoot tips (photo 1 F) have been acclimatised in greenhouse after storage period and plantlets have been formed in soil (**Photo 1 G**) with a survival frequency of 90%.

IV. Conclusions

This study proposes a simple cost-effective protocol for synthetic seeds production and short-term conservation and germplasm exchange of rare medicinal plants in Egypt. Shoot tips of 3-5mm in length were desirable for storage studies where viability and regeneration capacity were noted as most appropriate. Shoot tips encapsulation of *Aerva lanata* could be applied as an alternative method of propagation. The successful plant re-growth from the encapsulated shoot tips following low temperature (4C°) storage mostly depends on the kind of gel matrix, and the storage duration. Shoot tips could be storage for five months at 4C° without any

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subculture. The highest re-growth of *A. lanata* encapsulated shoot tips after five months of storage at 4C° reached 90%. Sodium alginate with 3% solution containing MS medium plus 3% sucrose and 100 mM calcium chloride can contribute to the development of high-quality beads with a high frequency of re-growth. This technology can also be considered an outstanding *in vitro* conservation strategy for Egyptian rare plants.

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