

# Alginate-encapsulated shoot tips and nodal segments in micropropagation of medicinal plants. A review

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## S u m m a r y

In recent years, the alginate-encapsulated *in vitro*-derived shoot tips and nodal segments have been employed as an alternative to somatic embryos method of artificial seed production. Encapsulation of non-embryogenic propagules offers an efficient technique for clonal propagation of elite genotypes e.g. plants of medicinal importance and also enables the establishment of basal collection for gene banks. 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. This paper presents a brief overview of current status of plants of medicinal value propagated by alginate-encapsulated non-embryogenic plant material.

**Key words:** *shoot tips, nodal segments, non-embryogenic micropropagules, artificial seeds, micropropagation, medicinal plants*

## INTRODUCTION

The idea of artificial seeds was proposed by Murashige [1], but first reports on their development were published a few years later.

The artificial seeds (also called somatic seeds, synthetic seeds, clonal seeds, synseeds, somseeds) are defined as an alternative to botanic seeds analogue consisting of somatic embryos surrounded by artificial coats. This definition, also

popular in these days, is based on the similarity of somatic embryos with zygotic embryos in morphology, physiology and biochemistry [2, 3]. A few years earlier Kamada [4] defined artificial seed as 'a capsule prepared by coating a cultured matter, a tissue piece or an organ which can grow into a plant body and nutrients with an artificial film'. This artificial seed concept comprised of 'an external film for strengthening the seed' which possibly implies the seed coat and 'an internal film for encapsulating nutrients required for growth of the cultured matter and plant hormones for controlling germination', a layer that probably simulates the endosperm tissue [5]. The currently used broader definition of synthetic seed is 'an artificially encapsulated somatic embryo, shoot or any other meristematic tissue which can develop into a plant under *in vitro* or *in vivo* conditions' [6].

The following two types of artificial seeds are known: desiccated and hydrated. The first type is produced from plant material either naked or encapsulated in polyoxyethylene glycol followed by its desiccation. In this context, the desiccated artificial seeds can be produced only in plant with desiccation-tolerant propagules. The hydrated artificial seeds are produced by encapsulating plant material in hydrogel coats. The second type of synseeds is produced in those species in which the propagules are recalcitrant and/or sensitive to desiccation [7].

This method has been employed as a suitable alternative for the use of somatic embryos [8] and became an important asset in micropropagation e.g. plants of medicinal value.

This paper describes the artificial seed technology, with a special focus on the potential use of propagules other than somatic embryos.

## ADVANTAGES OF ARTIFICIAL SEEDS

Artificial seeds have been proposed as a low cost propagation system of uniform plants. For this reason, it has been suggested as a powerful tool for mass propagation of elite species with high economic value (cereals, vegetables, fruit plants, ornamentals, aromatic and conifers) and rare or endangered taxons [7]. Among those, there are some studies describing propagation of species with medicinal value through encapsulated vegetative propagules (tab. 1). Efficient propagation, artificial seed production and storage protocols of threatened plants allow the continuous supply of plant material of medicinal importance [9] and enable the establishment of basal collection with representative genetic diversity for gene banks [10]. This technique is used for germplasm conservation of elite and unique species as well as products of wild hybridization and protected species on the verge of extinction. Artificial seeds may also be used for axenic, viable and elite germplasm preservation, particularly in recalcitrant species, as these seeds will not undergo desiccation [11].

The propagules encapsulation method is meant to be an alternative propagation of plants which can not be easily reproduced via generative seeds (transgenic seeds,

trees), non-seed producing species, polyploid plants with elite traits and male or female sterile ornamental hybrids. The synthetic seed technology can be also considered as an important tool for micropropagation either the cross pollinated crops since the allogamous nature makes it impossible to obtain elite clones by natural seeds [12]. In natural environment where various infections can threaten the organisms, it is important to produce virus-free plants protected against pathogens [13].

The significant advantage includes their designation as 'genetically identical materials'. The increasing utilization of synthetic seeds for clonal propagation necessitates assessment of genetic stability of conserved seeds and recovered plants [8, 14]. For measuring genetic uniformity of plants, DNA marker technology such as random amplified polymorphic DNA (RAPD) [15], flow cytometry [16] and other molecular analyses have been used successfully. The synthetic seed technology requires the inexpensive production with synchronous maturation.

Moreover, the technique of somatic seeds production offers space and equipment-saving option for storage of a wide range of plant materials at a low temperature [17]. This method also reduces the cost of labor as a result of no need for plant material transfer to the fresh medium [18]. Some other advantages of the synthetic seed technology are easy to handle due to small size of beads, higher scale-up capacity, possibility of automation of the whole production process and direct delivery to the field [10, 19]. The somatic seeds with vegetative propagules can be used for the exchange of axenic plant material between laboratories [19].

The artificial seeds method has also a potential for long-term storage in liquid nitrogen (cryopreservation) and mild- or short-term storage without losing viability, it also maintains the clonal nature of the resulting plants [17, 20-22].

One of the advantages of artificial seed production is that it is not subject to seasonal restrictions [23]. Finally, artificial seeds transportation between countries can be done without quarantine department permission.

## ARTIFICIAL SEEDS TECHNOLOGY

### Plant material

The artificial seed production technique involves suspending plant material (approximately 3-5 mm long) in a chemical solution. At the beginning somatic embryos were used as plant material for artificial seed production. During the last few years, considerable efforts have been made for encapsulation of non-embryogenic *in vitro*-derived plant material. This strategy was developed for plants in which the process of somatic embryogenesis has not been documented. The various types of unipolar vegetative propagules: microtubers, microbulbs, corms, rhizomes, microcuttings: shoots and nodal segments with apical or axillary buds; differentiating aggregates: organogenic callus and primordia or polar vegetative propagules: protocorms and protocorm-like bodies, can be used as explants for the preparation of synthetic

seeds [24]. There is a wide range of reports on the use of unipolar synthetic seeds from the last two decades. Among several non-embryogenic materials shoot tip explants are most responsive because of its mitotic activity in the meristem [25].

## Artificial seed coat

The matrix of capsules is responsible for the immediate surrounding of the plant material. Therefore, the matrix material has an important impact on the ultimate viability of the artificial seed. The artificial seed coat must: protect explants, be capable of including nutrients and other growth and biological factors, protect the formed artificial seed during storage and handling, incorporate a mechanism for activating 'germination', be non-toxic, compatible with the biological and chemical systems, should also be preferably biodegradable [5]. The nature of the soft hydrogel ensures that little pressure acts on the explant, and, therefore, minimizing harm to the plant material. In the hydrogel approach, the explants are mixed with a polymeric solution that when introduced drop-wise into a separate solution containing divalent metal ions, initiated a crosslinking reaction forming the hydrogel. The most common material used to generate this artificial endosperm is based on sodium alginate that forms a hydrogel in the presence of divalent calcium ions.

For the plant development the artificial endosperm should contain nutrients, mostly MS medium salts [26] and a carbon source [27]. According to Redenbaugh [28], the seed coat can be used as a reservoir of nutrients for development and survival of encapsulated propagules. In order to avoid bacterial contamination, the matrix can be supplemented with antibiotics [15]. A wide range of experiments of calcium alginate artificial seeds production have been published (tab. 1, references). The calcium alginate seeds were also coated with a synthetic polymer [29]. Such two-step procedures were also tested using polyethyleneimine [30] or chitosane [31] as a coating material. Several agents including sodium alginate with gelatin, potassium alginate, guar gum, agar, gerlite, carrageen, sodium pectate; polymeric materials such as potassium starch acrylamide, copolymer of potassium acrylate and acrylamide; and cellulose-based materials have been used for encapsulation [2, 5, 29]. The next innovation in artificial matrix was the concept of coating with polyethylene oxide homopolimers or acrylic copolymer [5, 32]. The plant material was also coated with a film composed of polyvinylchloride or polivinylacetate [32].

## Calcium alginate seed coat

For calcium alginate seed coat production, plant material is mixed with sodium alginate solution and dropped into calcium chloride solution. The beads containing e.g. shoot tips or nodal segments are held for 20–30 min in the calcium chloride solution in order to hardening of the seed coat. The major principle involved in the

alginate encapsulation process is that the sodium alginate droplets containing plant material when dropped into  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  solution form round and firm beads due to ion exchange between  $\text{Na}^+$  in sodium alginate and  $\text{Ca}^{2+}$  in calcium chloride. After the seed coat hardening, the calcium alginate seeds are thoroughly washed with sterile deionized water and transferred into water soaked lignin or selected medium. Alginate hydrogel is used as a matrix for synthetic seed production due to its moderate viscosity and low spinnability of solution, low toxicity for encapsulated explants as well as quick gellation, low cost and biocompatibility characteristics [11]. A gelling matrix of sodium alginate and calcium chloride solutions should be most suitable for formation of firm, clear and isodiametric beads. Concentration of both solutions influences not only texture, size and shape of artificial seeds but mainly rate of plantlet conversion. Low concentration of sodium alginate prolongs the polymerization duration and causes bead fragility, whereas application of high concentration results in seed coat hardness and shoot formation disturbance [33-35].

Addition of nutrients and plant growth regulators to the encapsulation solutions results in the increase of efficiency of viability of encapsulated plant material (tab. 1). The additives should have significant relationship with the efficiency of germination. In addition to prevent the explants from desiccation and mechanical injury, a number of useful materials such as nutrients, fungicides, pesticides, antibiotics and microorganisms may be incorporated into the encapsulation matrix. Sucrose is frequently used as a carbohydrate and energy source in plant tissue culture [36] and increases the tolerance of dehydration and maintains the tissue viability [20]. Sucrose is prone to promote the formation of adventitious and fibrous roots in plant development process [23]. It has been suggested that addition of activated charcoal improves the vigour of encapsulated plant material and its conversion into whole plant [5]. It can be explained by observation that addition of activated charcoal to the beads matrix results in absorbing the polyphenol exudates of the encapsulated propagules [37]. For the production of artificial seeds from apical shoot tips and axillary buds, plant material is usually at first treated with auxins for root induction and then the microcuttings are encapsulated in sodium alginate [7, 38]. Also for survival rate improvement after storage in low temperature, plant material was pre-treated with high abscisic acid concentration [5]. It was observed that decline in the plant recovery from encapsulated propagules, which were stored at low temperatures, may be due to the limited respiration through alginate matrix of encapsulated plant material [29, 35].

## STORAGE OF ARTIFICIAL SEEDS AND PLANT RECOVERY

*In vitro* cultures should be able to multiply plants efficiently, in this context especially species with medicinal value on the verge of extinction and preserve genetic stability of the conserved desired plant material e.g. with high secondary metabolites accumulation ability. Conservation of germplasm using artificial seeds can be envisaged either as short- and mild-term storage or as long-term cryopreservation

[39]. The artificial seeds may be carried out in different levels of time intervals. Encapsulated plant material is, in general, short-term stored for 6–12 months in closed sterile container at a temperature of 4°C. During long-term conservation in liquid nitrogen (–196°C) in different durations, cell division, metabolic and biochemical processes are arrested. For this reason the artificial seed matrix should be supplemented with nutrients playing an important role in storage (e.g. sucrose, salicylic acid, mannitol, paclobutrazol). Sucrose increases the tolerance to dehydration and maintains the tissue viability [20], salicylic acid plays a role during the plant response to abiotic stress such as low temperature [40], mannitol and paclobutrazol increase the tolerance of drought and cold stress [17]. *In vitro* conservation involves the maintenance of plant material in a pathogen-free environment. Long-term storage allows to maintain genes collections but storage in liquid nitrogen can not be applied to all genotypes. However, encapsulated in calcium alginate shoot tips and nodal segments of all genotypes can be stored at low temperature, but above 0°C. The aim is to increase the interval between subcultures by growth reduction for example by slow-growth maintenance under reduced temperature and light intensity, use of growth retardants (abscisic acid) and osmoticum (mannitol, sorbitol).

To summarize, the retention of biosynthetic potential of the encapsulated *in vitro*-derived plant material, implicates the use of this method for the storage of high secondary metabolites producing cells and tissue cultures for pharmaceutical purposes.

At the end of each storage period, synthetic seeds are immediately transferred to fresh germination medium and placed for the recovery of plantlets. After storage period the artificial seeds are re-grown under *in vitro* conditions on nutrient media supplemented or not with phytohormones for shoot development and root induction, followed by their hardening and growth in soil under *ex vitro* conditions. The alternative way for soil cultivation – artificial seeds are placed in soil pits in greenhouse, and then in ground [8-10, 12, 17, 19, 20, 23, 27, 33, 34].

## MEDICINAL PLANTS REGENERATION FROM ALGINATE-ENCAPSULATED EXPLANTS

The application of encapsulated shoot tips and nodal segments may contribute to the protection of rare and threatened medicinal plants. Although the artificial seed technique has been reported for more than two decades, for medicinal plants this method has not been developed sufficiently. The main limitations in conventional propagation of some species with medicinal value are: reduced endosperm, low germination rate and seedless varieties [11]. Many taxons also are desiccation-sensitive or have recalcitrant seeds, for this reason they can be stored only for limited time [21]. The above mentioned reasons indicate the need for the production of artificial seeds as a technique which combines the advantages of clonal multiplication with those of seed propagation and storage. To circumvent these problems, increasing attention has been paid to use encapsulated somatic embryos in clonal propagation

and short-term storage. However, encapsulation of somatic embryos was restricted mostly to medicinal plants in which somatic embryogenesis was documented [41]. Therefore, the encapsulation of non-embryogenic vegetative propagules like shoot tips and nodal segments of plants with medicinal value (tab. 1) also has been employed as a suitable alternative to micropropagation and plant material short-term storage [17, 20-22]. Despite this research, practical implementation of this technique encounters a number of difficulties associated with plant material maturation, development and regeneration. The parameters of encapsulation (mostly alginate coat matrix), suitable storage temperature and duration should be optimized for selected medicinal species to achieve a high percentage of plant conversion after conservation. It was shown that decline in the conversion of stored encapsulated propagules may be due to the inhibited respiration of plant material [29,35] or tendency of capsules to exsiccation under inadequate humidity conditions [28].

Table 1.

Studies on alginate-encapsulated propagules of selected medicinal plants

Plant species	Encapsulated plant material	Seed coat	References
<i>Allium sativum</i> L.	bulbets	1–4% SA, 0.2% AC + antibiotic , 225 mM CA,	15
<i>Ananas comosus</i> (L.) Merr.	axillary buds	3% SA, 82 mM CA	44
<i>Cannabis sativa</i> L.	axillary buds	2–6% SA in MS, 0.5 $\mu$ M TDZ, 2.5 $\mu$ M IBA, 25–100 mM CA	12
<i>Catalpa ovata</i> G. Don.	shoot buds	3% SA, 3% S, 50 mM CA	45
<i>Chonemorpha grandiflora</i> (Roth) S.M. & M.R.	shoot tips	3% SA in CaCl <sub>2</sub> -free ½ MS, 50 mM CA	46
<i>Cineraria maritima</i> L.	shoot tips, nodal segments	3% SA and 270 mM CA in ½ MS, 1.5% S	8
<i>Coffea arabica</i> L.	shoot buds	5% SA, 2% S, 100 mM CA	47
<i>Echinacea angustifolia</i> DC	shoots	5% SA, 50 mM CA	48
<i>Eclipta alba</i> (L.) Hassk	nodal segments	2–5% SA, 50,100,150 mM CA in water or MS	22
<i>Eryngium maritimum</i> L.	axillary buds	2–4% SA, 90 mM CA	49, 50
<i>E. campestre</i> L.			
<i>Gentiana pneumonanthe</i> L.	apical and axial shoot buds	3% SA in water (with or without 2.0 $\mu$ M IAA) or MS, 100 mM CA	9
<i>Gentiana scabra</i> Bunge	axillary buds	3% SA, 100 mM CA	51
<i>Glycyrrhiza glabra</i> L.	shoot tips, nodal segments	3% SA in MS, 100 mM	52
<i>Ocimum americanum</i> L.	axillary buds	2,4,6% SA in MS, 3%S, 100 mgL <sup>-1</sup> MI, 1.1–4.4 $\mu$ M BA, 630 mM CA	53
<i>O. basilicum</i> L.			
<i>O. gratissimum</i> L.			
<i>O. sanctum</i> L.			

Plant species	Encapsulated plant material	Seed coat	References
<i>Olea europea</i> L. cv. Moraiolo	microcuttings	2.5% SA in N, 1 mgL <sup>-1</sup> Zea, 5% S, 91 mM CA	54
<i>Phyllanthus amarus</i> Schum. and Thonn	shoot tips	1–5% SA in water or MS, 25–200 mM CA in water or MS	33
<i>Picrorhiza kurrooa</i> Royle ex Benth	shoot tips and nodal segments	3% SA in ½ MS, 1.5 S, 270 mM CA	55
<i>Plantago asiatica</i> L.	shoot tips	3–3.5% SA in MS, with or without 1.5–3.0% S, with or without 0.1 mgL <sup>-1</sup> BA	43
<i>Pseudostellaria heterophylla</i> (Miq.) Pax	micro-tubers	2.5% SA in ¼ MS, 1.0 mgL <sup>-1</sup> BA, 0.1 mgL <sup>-1</sup> NAA, 2% S, 180 mM CA	23
<i>Psidium guajava</i> L.	shoot tips	3% SA, 100 mM CA	21
<i>Punica granatum</i> L.	nodal segments	3% SA in MS, 100mgL <sup>-1</sup> MI, 4.44 μM BA, 0.54 μM NAA, 100 mM CA in MS	35
<i>Quercus</i> sp.	microcuttings	4% SA in N, 0.88μM BA, 126 mM CA	56
<i>Rauwolfia serpentina</i> (L.) Benth ex Kurz	shoot tips	3% SA, 100 mM CA	57
<i>Rauwolfia tetraphylla</i> L.	nodal segments	1–5% SA in MS, 100 mM CA in MS	58
<i>Rhodiola Kirilowii</i> Rgl. ex Maxim	axillary buds	5% SA, 50 mM CA	59
<i>Rubus chamaemorus</i> L.	axillary buds	5% SA, 50 mM CA	60
<i>Rubus idaeus</i> L.	shoot tips	3% SA in MS, 3.55 μM BA + 0.49 μM IBA, 270 mM CA	17
<i>Salvia officinalis</i> L.	shoot tips	2–3% SA in water, MS, ½ MS or 1/3 MS, 1.5% S, with or without 0.5–1.0 mgL <sup>-1</sup> GA <sub>3</sub> , 50 mM CA	61
<i>Simmondsia chinensis</i> (Link.) Schneider	shoot tips	2–5% SA, 100 mM CA	62
<i>Solanum nigrum</i> L.	nodal segments	2–4% in MS or water, 100 mM CA in water	41
<i>Spilanthes acmella</i> (L.) Murr.	shoot tips	1–5% SA in water, 3% SA in water (with or without 2.2 μM BA) or in MS, ½ MS, ¼ MS, 100 mM CA	10
<i>Stevia rebaudiana</i> (Bertoni) Bertoni	shoot tips	1–5% SA, 90–270 mM CA	63
<i>Trichosanthes dioica</i> Roxb.	shoot tips	3–5% SA and 12.5 mM CA in MS, 0.5–2.0 mgL <sup>-1</sup> BA or 0.5–2.0 mgL <sup>-1</sup> NAA or 1.0mgL <sup>-1</sup> BA + 0.5–2.0 mgL <sup>-1</sup> NAA	64
<i>Vitex negundo</i> L.	nodal segments	2–5% SA in MS, 25–200 mM CA	65
<i>Withania somnifera</i> (L.) Dunal	shoot tips	3–5% SA in MS or water, 25–200 mM CA in MS or water	34
<i>Zingiber officinale</i> Rosc.	microshoots	4% SA in MS, 2.5mgL <sup>-1</sup> BA, 100 mM CA	66

(SA – sodium alginate, CA – calcium chloride, AC – activated charcoal, MS – Murashige & Skoog medium, BA – 6-Benzyladenine, NAA –  $\alpha$ -Naphthaleneacetic acid, S – sucrose, MI – myo-inositol, M – mannitol, N – nutrients from varied media)



Our previous experimental studies revealed that plant *in vitro* cultures and biotechnology give an opportunity to carry out the phytochemical investigations of rare, vulnerable and endangered medicinal plants with high content of desired bioactive compounds without collecting them from natural localities [42]. For *in vitro* germplasm storage of selected Polish rare and endangered species rich in secondary metabolites, alginate encapsulated vegetative propagules were applied [Figure 1].

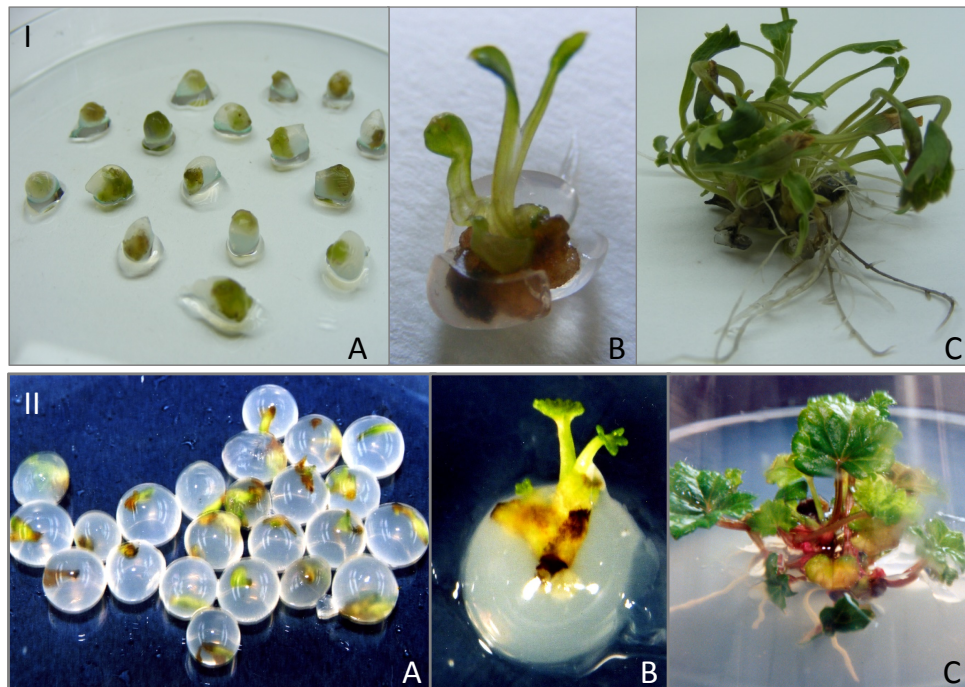


Fig. 1.  
Plantlet regeneration from encapsulated axillary buds of *Eryngium maritimum* L. (I) and *Rubus chamaemorus* L. (II) A- axillary buds encapsulated in calcium alginate beads, B – shoot emergence from encapsulated axillary buds, C- plant with well-developed shoots and roots

## DISADVANTAGES OF ARTIFICIAL SEEDS

Despite the advantages of the artificial seed technology, it is somehow limited. Among numerous studies on artificial seeds, some authors reported:

- lack of dormancy and stress tolerance in artificial seeds that limit the storage
- lack of synchrony in plant material development

- improper maturation and poor conversion into plantlets [7]
- storage at low temperature significantly reduces the viability and plant recovery [43].

## CONCLUSION

The artificial seed technology provides an alternative method of micropropagation for a wide range of medicinal plants, especially desirable elite genotypes. However, successful plant retrieval from encapsulated vegetative micropropagules following short-term storage is mostly depends on plant species, matrix composition and period of storage. Moreover, the artificial seed technique combines the advantages of clonal multiplication with those of seed propagation and storage. The encapsulated non-embryogenic propagules have become a potentially cost-saving technique for clonal propagation and may be used as an alternative to the artificial seeds with somatic embryos. The *in vitro* encapsulated shoot tips and nodal segments technology facilitates conservation of genetic diversity and germplasm of wide spectrum of medicinal plants through cryopreservation.

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## WIERZCHOŁKI PĘDÓW I ODCINKI WĘZŁOWE OTOCZKOWANE W ALGINIANIE JAKO ALTERNATYWNA METODA MIKORozmnażania Roślin Lecznicych

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### Streszczenie

Wierzchołki i odcinki węzłowe pędów pochodzące z kultur *in vitro*, zamknięte w alginianowych kapsułkach od ponad dwóch dekad wykorzystywane są jako alternatywna – w stosunku do zarodków somatycznych – metoda wytwarzania sztucznych nasion. Kapsułkowanie materiału rozmnożeniowego, innego niż zarodki somatyczne oferuje możliwość rozmnażania klonalnego i krótkoterminowego przechowywania roślin. Technika ta stosowana jest wobec gatunków, dla których nie opracowano metody embriogenezy somatycznej np. elitarnych genotypów roślin o właściwościach leczniczych. Metoda kapsułkowania wierzchołków i odcinków węzłowych pędów umożliwia tworzenie podstawowej kolekcji dla banków genów. Artykuł ten prezentuje przegląd roślin leczniczych rozmnażanych za pomocą wegetatywnych propaguli kapsułkowanych w alginianie.

**Słowa kluczowe:** wierzchołki pędów, odcinki węzłowe pędów, wegetatywne mikropropagule, sztuczne nasiona, mikrorozmnażanie, rośliny lecznicze