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Encapsulation in Calcium Alginate of Nodes from Stolons of *Mentha spicata* L.

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Abstract: It is well known that the products of encapsulation (multifunctional beads and synthetic seeds) can be used as innovative technological tools to integrate micropropagation both for plant germplasm conservation and to simplify the management of propagation materials in nurseries. Nevertheless, the usual concept of encapsulation concerns the use of initial in vitro derived explants. In this study, for the first time, in vivo derived organs of *Mentha spicata* L., obtained through the excision of fragments (nodes) from stolons of cultivated mother plants, were employed. The artificial endosperm had a tenfold reduced concentration of Murashige and Skoog (MS) substrate, with the addition of sucrose (5 g L^{-1}), 6-benzyl-aminopurine (BAP) (0.1 mg L^{-1}) and 1-naphthalene acetic acid (NAA) (0.01 mg L^{-1}). Moreover, the calcium alginate matrix was enriched with different thiophanate-methyl (TM) concentrations (0, 10, 50, 100 and 200 mg L^{-1}) in order to prevent possible contamination during the conversion in nonsterile conditions. Interesting results were obtained encapsulating every single node of fresh stolon as a bipolar propagule able to develop a whole plantlet (conversion), as the coated seed in other species. The synthetic seeds of spearmint without TM in the artificial endosperm showed a satisfactory ability to convert (56.7%) into plantlets after sowing in soil under nonsterile conditions. TM at 100 and 200 mg L^{-1} negatively affected the total emergence, which decreased to 30.0 and 33.3%, respectively. In general, in the artificial seeds without TM, higher values for most of the aboveground and belowground plants parameters were recorded compared to naked nodes.

Keywords: spearmint; synthetic seed; conversion; bipolar propagule; plantlets

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1. Introduction

Mentha spicata L. is an aromatic perennial herb belonging to the *Lamiaceae* family. Spearmint is native to Europe and South East Asia, and is distributed mainly in the temperate and subtemperate regions of the world. It is an industrial crop that is widely cultivated as a source of essential oils for flavoring extraction, and the nutraceutical and cosmetic industries [1]. *Mentha spicata*, in addition to a root system that consists of bundles of small roots, is equipped with stolons, very vigorous vegetative organs used by the plant to expand and propagate itself [2], as well as acting as reserve organs. In particular, each stolon has knots characterized by the ability to develop both roots and shoots. Indeed, each stolon node, if isolated, can act as a bipolar propagule, capable of developing a complete plantlet. This type of structure is ideal for the encapsulation technology aimed at obtaining synthetic seeds. Encapsulation technology has been studied for many years and applied to numerous species, consisting of covering an explant with a nutritive and protective matrix that is only equipped with the vegetative pole or even the root pole. In this second case, the final product is a synthetic seed capable of developing a complete plantlet [3–5]. Most of the experiments conducted on the use of encapsulation to obtain products that can be used for the production of nursery material, to simplify the diffusion and exchange of plant

germplasm between laboratories, for short-term storage or medium–long-term storage periods, were almost always carried out using in vitro derived propagules [4,6,7]. This work aimed to evaluate for the first time the effect of the encapsulation on in vivo derived propagules, such as spearmint stolon nodes, to obtain synthetic seeds without the necessity for sterile conditions for propagation, sowing them directly in the greenhouse.

2. Materials and Methods

Preliminarily, spearmint mother plants were taken from the field, paying attention to collect the root systems and limiting any damage to them (Figure 1).

Figure 1. Plants of *Mentha spicata* were taken from the field to be used in the experiment.

The stolons were separated from the plants' epigeal parts and the bundles of capillary roots. Before being used in the laboratory, stolons were washed in running water to remove soil and other impurities. At the beginning of the experiment, each stolon was isolated in portions of 3–5 mm in length (propagules) containing one node each one. Subsequently, the nodes were subjected to surface disinfection under sterile conditions using a horizontal laminar flow cabinet. At the beginning, the propagules were immersed in an antioxidant solution containing ascorbic acid (100 mg L^{-1}); then they were immersed in an ethanol solution (65%) for 20 s and finally, the material was subjected to two rinses in sterile distilled water (Figure 2).

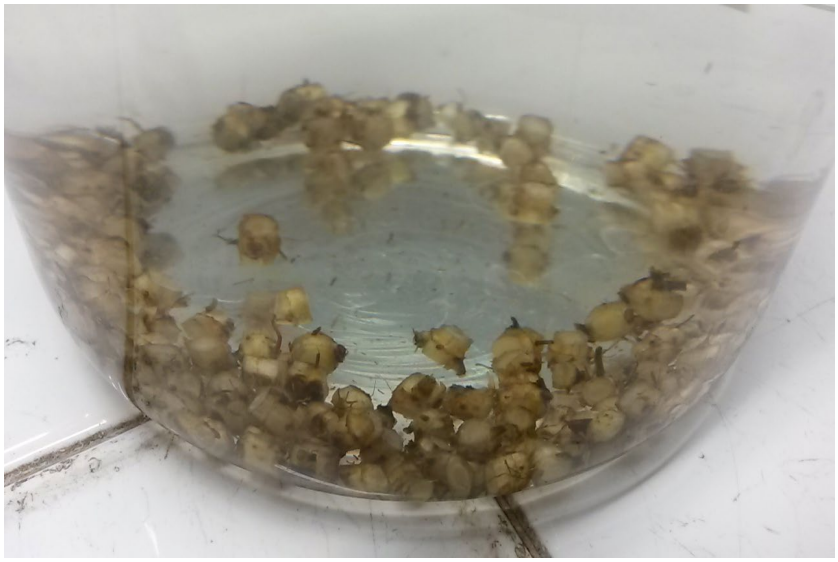

Figure 2. Isolated nodes of stolon during the surface sterilization.

Encapsulation was carried out according to the protocol suggested by Standardi and Micheli [4]. In brief, the procedure consists of three steps: (i) coating: the explants were immersed in an encapsulating solution containing the nutritive MS components (artificial endosperm) and sodium alginate (25 g L^{-1}) for a few seconds, (ii) complexation: the alginate-coated explants were dropped into a complexation solution containing the artificial endosperm enriched with calcium chloride (11 g L^{-1}) for 35 min; (iii) rinsing: the coated explants were washed in distilled water in order to remove the toxic residual ions of chloride and sodium.

The artificial endosperm had a tenfold reduced concentration of Murashige and Skoog (MS) substrate [8], with the addition of sucrose (5 g L^{-1}), 6-benzyl-aminopurine (BAP) (0.1 mg L^{-1}) and 1-naphthalene acetic acid (NAA) (0.01 mg L^{-1}), and pH = 5.7. The calcium alginate matrix was enriched with different concentrations (0, 10, 50, 100 and 200 mg L^{-1}) of fungicide thiophanate-methyl (cod. 45688, Sigma-Aldrich, St. Louis, MO, USA) in order to sow the synthetic spearmint seeds directly in the greenhouse and prevent possible contamination during the conversion in nonsterile conditions. The sowing was carried out on a bench of an unheated greenhouse, using alveolar polystyrene containers, filled with a substrate consisting of sand and peat (1:3, v:v). A layer of agriperlite was distributed under the polystyrene containers to avoid water stagnation. Thirty synthetic seeds (3 replications of ten propagules each) for each treatment and thirty naked nodes were sown. The irrigation was carried out every three days with tap water. Starting from the 15th day up to the 56th day after sowing, the emergence of spearmint plantlets was periodically monitored. Subsequently, the experiment continued for another 2 months to allow the complete development of all emerged plantlets. At the end of the experiment, both the above-ground (sprouting (or emergence) (%), basal shoots (n), length of basal shoots (mm), nodes on basal shoots (n), lateral shoots (n), length of lateral shoots (mm), nodes on lateral shoots (n)) and belowground (rooting (%), stolons (n) and length of stolons (mm)) parameters were measured to evaluate the synthetic spearmint seeds' performance. Collected data were subjected to analysis of variance (ANOVA), according to a randomized design and significant differences were assayed by the Tukey's test, using the SISVAR statistical program.

3. Results

The results reported in Table 1 show that the greatest emergence of new shoots, in general, was concentrated within the first 15 days (19.4%).

In particular, the synthetic seeds characterized by a TM-free artificial endosperm (TM0) showed the highest number of sprouted buds at the first monitoring and the most elevated TE after 56 days. The surveys after the 15th day showed lower values of PE, never higher than 3.3%. After 56 days from sowing, similar percentages of emergence were recorded for naked propagules and synthetic seeds containing intermediate doses of thiophanate-methyl (TM10 and TM50), while the fungicide used at 100 and 200 mg L^{-1} negatively affected TE (Table 1).

The other above-ground parameters monitored at the end of the experiment are reported in Table 2.

The shoots developed from pre-formed buds at each propagule corresponded to the main ones obtained during the sprouting and were called "basal shoots". In contrast, "lateral shoots" were identified as the secondary ones developed from adventitious buds in correspondence of the same stolon node (Figure 3).

Table 1. Emergence of naked nodes and synthetic seeds of *Mentha spicata* monitored between the 15th and the 56th day from sowing.

Emergence (Days)	Naked [1] (n)	TM0 [2] (n)	TM10 [2] (n)	TM50 [2] (n)	TM100 [2] (n)	TM200 [2] (n)	PE [3] (%)
15	5	12	6	3	4	5	19.4 a
20	3	1	2	0	0	0	3.3 b
24	1	1	0	2	0	0	2.2 b
27	3	0	0	0	1	0	2.2 b
31	0	1	1	1	2	1	3.3 b
34	1	0	0	1	1	1	2.2 b
38	0	1	0	1	0	1	1.7 c
41	0	0	4	0	0	1	2.8 b
45	0	0	0	0	0	0	0.0 d
48	0	0	0	4	0	0	2.2 b
52	1	1	0	0	0	1	1.7 c
56	0	0	0	0	1	0	0.6 d
TE [4] (%)	46.7 b	56.7 a	43.3 b	40.0 b	30.0 c	33.3 c	

Different letters within the PE column or the TE line indicate significant differences according to the Tukey's test ($p \leq 0.05$). [1] Naked = nodes of stolon not encapsulated. [2] The artificial endosperm of each synthetic seed was enriched with thiophanate-methyl at 0 mg L^{-1} concentration (TM0), 10 mg L^{-1} (TM10), 50 mg L^{-1} (TM50), 100 mg L^{-1} (TM100) and 200 mg L^{-1} (TM200). [3] PE = total emergence periodically monitored. [4] TE = total emergence (sprouting) monitored at the end of the experiment.

Table 2. Above-ground parameters monitored at the end of the experiment.

	Basal Shoots (n)	Basal Shoot Length (mm)	Basal Shoot Nodes (n)	Lateral Shoots (n)	Lateral Shoot Length (mm)	Lateral Shoot Nodes (n)
Naked [1]	2.7 a	60.1 b	9.0 b	6.7 a	15.3 c	4.3 b
TM0 [2]	2.3 b	71.1 a	9.7 a	6.2 a	17.5 b	4.8 a
TM10 [2]	1.9 c	66.3 a	9.8 a	4.8 b	24.9 a	5.4 a
TM50 [2]	2.8 a	47.8 c	8.9 b	3.3 c	22.2 a	4.6 b
TM100 [2]	2.4 b	60.6 b	8.4 c	3.3 c	16.4 c	3.9 b
TM200 [2]	2.2 b	58.4 b	9.0 b	2.6 c	20.0 b	4.9 a

Different letters within each column indicate significant differences according to the Tukey's test ($p \leq 0.05$). [1] Naked = nodes of stolon not encapsulated. [2] The artificial endosperm of each synthetic seed was enriched with thiophanate-methyl at 0 mg L^{-1} concentration (TM0), 10 mg L^{-1} (TM10), 50 mg L^{-1} (TM50), 100 mg L^{-1} (TM100) and 200 mg L^{-1} (TM200).

Figure 3. A basal shoot (on the **left**) and some lateral shoots (on the **right**) developed from synthetic seeds of *Mentha spicata*.

As regards the development of the "basal shoots", the best results were expressed by naked nodes (2.7) and synthetic seeds encapsulated with 50 mg L^{-1} of fungicide (TM50) (2.8). A lower number of basal shoots in TM0 and TM10 corresponded to the highest average length (respectively, 71.1 and 66.3 mm) and the number of new nodes (9.7 and 9.8). Even in the case of "lateral shoots", the fungicide-free (TM0) naked nodes and synthetic seeds developed the highest average number of shoots (6.7 and 6.2), although the greater length of new shoots was shown by synthetic seeds with lower concentrations of fungicide (24.9 mm with TM10 and 22.2 mm with TM50). As regards the number of nodes, the highest value was recorded in TM10 (5.4) and the lowest in TM 100 (3.9).

The natural habit of the spearmint is predominantly semi-prostrate. In this experiment, it was considered important to evaluate if encapsulation could influence this behavior. The results (data not shown) proved that the semi-prostrate habit seems to be correlated to the length of the shoots produced. Indeed, the treatments with the highest incidence of this habitus (respectively, 50.0% in TM0 and 61.5% in TM10) corresponded to those with the longest basal shoots (Table 2).

The use of bipolar propagules (such as stolon nodes) allows the development of a complete plantlet with shoots and a root system: this type of development is called "conversion" in synthetic seeds (it corresponds to the "germination" of gametic seeds). Therefore, to complete the study, the development of the belowground organs was monitored, as reported in Table 3.

Table 3. Hypogeous parameters monitored at the end of the experiment from each node.

	Rooting (%)	Conversion (%)	Stolons (n)	Stolon Length (mm)
Naked [1]	46.7 b	46.7 b	0.8 c	93.6 a
TM0 [2]	56.7 a	56.7 a	1.2 a	94.5 a
TM10 [2]	43.3 b	43.3 b	0.5 c	96.7 a
TM50 [2]	40.0 b	40.0 b	0.7 c	46.3 c
TM100 [2]	30.0 c	30.0 c	1.0 b	73.2 b
TM200 [2]	33.3 c	33.3 c	0.5 c	48.0 c

Different letters within each column indicate significant differences according to the Tukey's test ($p \leq 0.05$). [1] Naked = nodes of stolon not encapsulated. [2] The artificial endosperm of each synthetic seed was enriched with thiophanate-methyl at 0 mg L^{-1} concentration (TM0), 10 mg L^{-1} (TM10), 50 mg L^{-1} (TM50), 100 mg L^{-1} (TM100) and 200 mg L^{-1} (TM200).

All the propagules showed full conversion (Figure 4, left): indeed, all those sprouted (Table 1) had also rooted (Table 3). The best rooting and conversion level (56.7%) was detected in encapsulated propagules in the absence of fungicide (TM0), while the higher fungicide concentration (from TM10 to TM200) limited the synthetic seeds' rhizogenic ability. Rooting could have been better assessed by monitoring the length of individual roots, but this was not possible due to the abundance of roots from each node (Figure 4, right). Rather, stolon production (Figure 4, right), as the propagation organs of the species, is very interesting.

Indeed, the highest average number of stolons developed by each node was recorded in TM0 while the greatest stolon length was registered on naked nodes, followed byTM0 and TM10 (Table 3). This demonstrates that encapsulation did not affect the morphology and functionality of the development of spearmint plantlets, whereas only the fungicide presence in the artificial endosperm reduced rooting and conversion percentages, stolon numbers and length.

Figure 4. Conversion (**left**) and development of abundant roots and new stolons (**right**) from synthetic seeds of *Mentha spicata*.

4. Discussion

The main aim of the present study was to verify the possibility of achieving conversion from nodes of spearmint stolon, excised directly from cultivated plants, encapsulated in calcium alginate, and sown in the greenhouse in a substrate consisting of soil and peat rich in biotic and abiotic factors potentially antagonistic to the development of the young plantlets.

According to the literature, the methodological novelty of this work was represented by the use of in vivo derived propagules (stolon nodes) of *Mentha spicata*. Each node of the stolon is a bipolar propagule able to develop both aboveground organs (buds and shoots) and belowground organs, through enough nutritive reserves essential for the development of a whole plantlet. In addition, it is well known that the majority of synthetic seeds are produced from encapsulated in vitro derived propagules; however, the possibility of encapsulating in vivo derived ones has been confirmed in some plant species [4,9–11].

Analyzing the results of this experiment, in general, it appears that the encapsulated nodes showed a faster emergence, especially within the first 15 days from sowing. The artificial endosperm of the encapsulating matrix played a decisive role through its nutritive and protective function, by stimulating the conversion and limiting possible mechanical damage to the vegetative and rooting poles in each node. It should be obvious to expect that the artificial endosperm supports an in vitro derived explant during its development, as it is a less vigorous material than a stolon node directly taken from a plant cultivated in the field (in vivo derived). However, the in vivo derived explants benefit from the nutritive substances supplied by the mother plant, at least until the moment of its excision but only a small part of the nutritive substances are stored in the stolon node. In our experiment, the nutritive function of the artificial endosperm proved effective in supporting the development of new plantlets from the encapsulated nodes. The composition of the endosperm used in this experiment was similar to that used by Islam and Bari in *Mentha arvensis* [12], although slightly modified considering the different types of propagules, and

it seemed to assure a high level of conversion of the encapsulated nodes in comparison to the naked ones.

In contrast, the addition of a fungicide to the artificial endosperm did not seem to have any positive effect on the sprouting (emergence) at least compared to the control treatment (TM0). It is clear that the use of thiophanate-methyl was irrelevant under the experimental conditions applied, since a sowing substrate consisting of a mix of sand and peat (1:3, v:v) and tap water for irrigation in the greenhouse was used, in an environment not entirely controlled from a phytosanitary point of view and in the absence of exogenous pesticide treatments. The indirect control of pathogens in the absence of the fungicide (TM 0) could also be due to the conversion precocity, concentrated within the first 15 days. As a consequence, the use of fungicide for the control of any pests was probably unnecessary, because thiophanate-methyl at higher concentrations also seemed to affect the performance of the synthetic spearmint seeds. This result is different from that reported by Germanà et al. [13] by using in vitro derived somatic embryos of *Citrus reticulata* Blanco. However, in general, the difficulties of sowing artificial seeds directly in soil or in commercial substrates under nonsterile conditions are considered one of the main limitations of the practical use of this technology [11,14–17]. There are studies only partly similar to the one described. Under non-aseptic conditions, Bapat and Rao [18] encapsulated in vitro derived axillary buds of *Morus indica* in an autoclaved alginate matrix containing either of the fungicides carbendazim, benomyl and bavistin to prevent contamination when sown in soil. Lata et al. [19] studied in vivo conversion placing synthetic seeds of *Cannabis sativa* in pots containing 1:1 potting mix-fertilome with natural coco growth medium moistened with full strength MS medium, supplemented with PPM. Encapsulated somatic embryos of *Paulownia elongata* were sown in vials containing peat and perlite (3:1) and incubated under growth chamber conditions to evaluate their conversion after 5 weeks [20].

Moreover, synthetic seed technology has been successfully employed in various medicinal and ornamental plants but to the best of our knowledge always starting from in vitro and not in vivo explants. For instance, synthetic seeds were obtained also from Fonseca et al. [21] encapsulating the nodal segment of the black-oil tree (*Celastrus paniculatus* Willd), a medicinal plant grown in vitro. The beads germinated with 2 mg L^{-1} BAP and 0.2 mg L^{-1} NAA provided an 80% in vitro germination percentage. A protocol for synthetic seed production in jojoba using axillary buds from established cultures in vitro was developed by [22]. The plantlet conversion efficiency was highest in synthetic seeds developed with 3.0% sodium alginate and 100 mM Calcium chloride with 40 g L^{-1} sucrose in MS medium. This combination gave the earliest bud initiation with the maximum number of shoots per explant and shoot length. Synthetic seed production of *Enicostema axillare* (Lam.) A. Raynal., a medicinal plant, through nodal and root explants as attempted by [23]. The encapsulated nodal explants germinated and produced multiple shoots in 2 mg L^{-1} BAP and kinetin (KIN) 0.5 mg L^{-1} in combination with 2 mg L^{-1} gibberellic acid (GA_3). Moreover, the synthetic seed prepared using $\frac{1}{4}$ strength MS medium and stored at 18 °C were preserved for up to 80 days and more without losing germination ability (80%). An efficient synthetic seed production protocol was established by [24] for the conservation of *Decalepis salicifolia* (Bedd. ex Hook.f.) Venter, an endemic and critically endangered ethnomedicinal species. In this case, shoot tip and nodal explants were encapsulated in sodium alginate and their regeneration was achieved following storage at 4 °C for up to 12 weeks. Successful rooting was obtained in modified MS medium with low nitrate and high sucrose concentration. The in vitro derived rooted plants were successfully hardened and established in the field with 100.0% survival rate.

Explants of shoot tips and first-node segments of *Viburnum dentatum* L., excised from in vitro derived viburnum microshoots, were encapsulated in 2.5% sodium alginate mixed with liquid MS nutrient medium and hardened in 50 mM of calcium chloride producing solid, soft and uniform beads. Increasing the calcium chloride concentration to 100 mM, the beads obtained were firm and of a uniform globular shape, suitable for handling, and exhibited a germination response of 48.9% [25].

5. Conclusions

The present experiment allowed us to verify that it is possible to encapsulate in vivo derived stolon nodes of *Mentha spicata* and that the synthetic seeds obtained are also capable of converting in in vivo conditions on substrates similar to those used in nurseries. The speed of conversion allowed us to control pest development. The developed plantlets retained all the morphological and functional characteristics of the mother plants.

Other interesting aspects to study could be the optimization of the synthetic seeds' conversion levels; improving the vegetative activity of the plantlets, i.e., by using biostimulants; the verification of the possibility to store the synthetic seeds of *Mentha spicata* for conservation of plant germplasm; and the simplification of plant management in the nursery. All these achievements could allow the diffusion of the encapsulation technique to improve the propagation and cultivation of this species.

However, it would be indispensable to maximize the efficiency of the procedures, possibly by using automation systems, which, in both synthetic seed preparation and sowing phases, would make it possible to drastically reduce the incidence of nursery production costs.

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