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ORIGINAL RESEARCH PAPER

An improved encapsulation protocol for regrowth and conservation of four ornamental species

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Abstract
The encapsulation technology, initially developed for clonal propagation through the production of synthetic seeds with somatic embryos, is currently proposed for use with non-embryogenic explants, such as buds and nodal segments (unipolar propagules). In the present study, the encapsulation procedure and its effect on shoot regeneration were evaluated. Apical buds isolated from shoot cultures of four ornamental species (*Photinia* × *fraseri* Dress., *Polygala myrtifolia* L., *Metrosideros excelsa* Soland. ex Gaertn., and *Rosa*) were encapsulated in 3% sodium alginate. Effects of complexation time, sucrose concentration, and storage temperature on the regrowth ability of propagules were assessed. With the appropriate combination of sucrose concentration and polymerization time, the encapsulated explants proved to have a better regrowth (80–100%) after sowing than the naked ones. In addition, medium-term storage of *Metrosideros* encapsulated explants promoted a high level of regrowth (74%) after 4 months in the dark at 10°C; while polygala beads were preserved up to 8 months regardless of storage temperatures. Potential current applications of encapsulation technology and the future use of beads in vivo conditions are also discussed.

Keywords
alginate; bead; regrowth; synthetic seed; preservation

Introduction

The quality of plant material is essential for increasing the efficiency of multiplication methods of elite superior varieties, and the use of biotechnological approaches represents an effective support to yield high-quality horticultural productions. In the ornamental plant market, where large scale propagation is often subject to external business factors that affect sale price, in vitro cultures could be used to lower production costs and so encourage economic benefits [1].

Encapsulation has been recently proposed as plant biotechnology in fruit, forestry, horticultural, medicinal, and aromatic species [2–5]. In addition, the technology is very promising with regard to improving the micropropagation and in vitro conservation of ornamental plants [6,7], especially if applied commercially on a large scale. Furthermore, encapsulation could be an important tool for mass propagation, especially for species or varieties characterized by low proliferation rates, limited rooting, and difficult adaptation during acclimatization. Moreover, this technology can be used to propagate disease-free clones and to preserve gene pools. Originally, encapsulation

was applied to somatic embryos, to simulate the formation and germination of natural seeds. As a result, Murashige [8] proposed the term of "artificial seed", also referred to as "synthetic seed" or "synseed". Since then, the technology has expanded to include the use of other in vitro-derived explants in "artificial endosperm" [9]. Over time, the concept of synthetic seeds has evolved, resulting in a new definition: "artificially encapsulated somatic embryos, shoots or other tissues which can be used for sowing under in vitro or ex vitro conditions" [10] and also "that may maintain the potential germination also after storage" [11]. Currently, it is well accepted that synthetic seed can contain any kind of non-embryogenic explants from tissue culture, such as axillary buds, shoot tips, nodal segments, bulblets, protocorms, portion of callus, naturally able (bipolar propagule) or induced (unipolar propagule) to evolve into whole plantlets [12]. Effective protocols for preparing synthetic seeds with non-embryogenic explants have been reported for propagating and conserving fruit species [13,14] and ornamental species [15–18].

Differently from synthetic seed, the development of an encapsulated non-induced unipolar propagule consists of the breakage of alginate matrix and extrusion of at least one small shoot. This behavior is peculiar to the bead, which can be defined as "an encapsulated portion of in vitro-derived plant tissue possessing the ability to evolve in shoot (not into a whole plantlet)" [12]; this capacity is called "regrowth" rather than "germination" used by many other authors, in spite of the absence of direct root regeneration [19,20].

Beads can be useful for the exchange of germplasm of elite genotypes and axenic plant material between laboratories due to the small size and relative ease of handling [21]. Moreover, the most recent application of this technology involves the use of encapsulated propagules in medium and long-term storage [22]. Particularly, medium-term conservation aims to increase the interval between subcultures, reducing the growth of the plant material [23]. On the other hand, successful long-term conservation can be achieved when plant material is stored at ultra-low temperature as in liquid nitrogen [24]. Among the cryogenic technologies available today, two in particular are used with synthetic seeds: encapsulation-dehydration and encapsulation-vitrification [25–27].

The ornamental species object of our investigation (fraser photinia, polygala, metrosideros, and two roses) are economically important plants for commercial laboratories and the present research reports the development of suitable encapsulation protocols using apical buds with the aim to improve the regrowth of beads. For this purpose, different concentrations of sucrose in alginate matrix and duration of complexation times, in order to produce ideal beads, were assessed. Moreover, a medium-term conservation protocol was studied as convenient option in commercial micropropagation laboratories which need today to widen the offer of species and cultivars and achieve to better organization of their in vitro and ex vitro productions.

Material and methods

Explant source

Shoots of ornamental plants, fraser photinia (*Photinia* × *fraseri* Dress.), polygala (*Polygala myrtifolia* L.), metrosideros (*Metrosideros excelsa* Soland. ex Gaertn.) and two roses, 'Domenica', a white Rose Cityflor selection and 'S. Antonio', an ancient hybrid tea rose, were used. All shoots were subcultured on a suitable proliferation medium. Fraser photinia shoots were transferred monthly on QL (Quoirin and Lepoivre) [28] medium supplemented with 4.4 µM 6-benzyladenine (BA), metrosideros and rose shoots on MS (Murashige and Skoog) [29] with 0.88 µM and 2.0 µM BA, respectively; polygala shoots were subcultured on DKW (Driver and Kuniyuki) [30] medium with 8.8 µM BA. All media contained 30 g/L sucrose and 0.7% agar and were adjusted to pH 5.8 before autoclaving. The cultures were maintained in 500-mL glass jars in a growth chamber at 23 ±1°C, under a 16-h photoperiod and 60 µmol m^{-2} s^{-1} photosynthetically active radiation provided by cool-white fluorescent lamps (standard culture conditions).

Encapsulation and preparation of beads

Apical buds with two or four primordial leaves (1.5–2.0 mm) were excised from well-proliferated shoots and were encapsulated using sodium alginate as the gelling matrix and calcium chloride as the complexing agent. Each apical bud was plunged in nutritive solution, as artificial endosperm, composed of 3% sodium alginate (w/v) and MS basal medium, and supplemented with two sucrose concentrations (30 or 60 g/L). Drops of encapsulating matrix containing explants were then transferred into the MS basal medium supplemented with 100 mM calcium chloride ($CaCl_2 \cdot 2H_2O$) to obtain encapsulated buds, hereafter called beads. Different times (15, 20, 30, and 40 min) of contact with the complexing solution were tested to achieve polymerization of the sodium alginate and to obtain suitable beads with uniform texture. After each tested period, the encapsulated buds were retrieved and rinsed three times in sterile double-distilled water to remove traces of $CaCl_2$.

Regrowth ability and average regrowth time

In order to recover shoots, encapsulated apical buds were immediately transferred to Petri dishes (Ø 90 mm) in appropriate sowing media as the above-mentioned proliferation media. Five Petri dishes, each containing 10 beads per genotype, were placed in a growth chamber at 23 ±1°C, under a 16-h photoperiod. The regrowth ability was determined when the shoot extrusion was detected through the calcium alginate matrix breakage, as suggested by Standardi and Micheli [12]. Only shoots at least 5 mm long were considered suitable for data collection. The average regrowth time of beads was calculated as follows: Σ (*NxTx*) / number of developed shoots, where *Nx* is the number of developed shoots within consecutive intervals of time, and *Tx* the number of days between the beginning of the test and the end of the specific interval of time. The regrowth of naked apical buds (i.e., non-encapsulated) was also evaluated for each species.

Medium-term conservation

After 20 min polymerization time, encapsulated apical buds of polygala and metrosideros were stored at two temperatures (4 and 10°C) in darkness. The beads were maintained on species-specific agarized hormone-free medium in Petri dishes. Four different cold storage periods (2, 4, 6, and 8 months) were evaluated for conservation. Following each storage period, 45 beads were transferred to a proliferation medium in standard culture conditions. The effectiveness of medium-term conservation was evaluated after 6 weeks of culture, and the percentage shoot regrowth was recorded.

Direct regrowth in ex vitro conditions

For the regrowth in ex vitro conditions, apical buds of photinia and metrosideros were encapsulated with 3% Na-alginate and 100 mM $CaCl_2 \cdot 2H_2O$ treatment for 20 min. A hundred beads were soaked in plastic pots (each 14 cm^3) containing sterile mixture of peat and perlite (1:1). The trays were placed and kept inside a plastic transparent box (46 × 20 × 10 cm) with adjustable opening for moisture exchange (Fig. 1). Plastic boxes were maintained in a phytotron, at 24 ±1°C, 16-h light period, and 60 μmol m^{-2} s^{-1} light intensity, to test the emergence of shoots from beads. The regrowth percentage was recorded weekly.

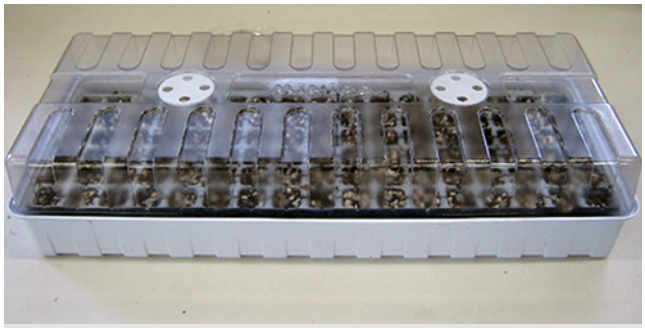

Fig. 1 Plastic box containing beads sown in ex vitro conditions.

Data collection and statistical analysis

Regrowth ability of encapsulated explants was evaluated in terms of the percentage of apical buds able to break

Tab. 1 Effect of polymerization times and different sucrose concentrations on regrowth of encapsulated apical buds.

Polymerization time (min)	Sucrose (g/L)	*Photinia* × *fraserii*		*Polygala mirtifolia*		*Metrosideros excelsa*		Rose 'Domenica'		Rose 'S. Antonio'	
		regrowth (%)	average regrowth time (days)	regrowth (%)	average regrowth time (days)	regrowth (%)	average regrowth time (days)	regrowth (%)	average regrowth time (days)	regrowth (%)	average regrowth time (days)
15	30	50.0 ±1.5 [b]	14	86.7 ±1.3 [a]	12	46.7 ±1.2 [b]	21	90.0 ±0.5 [a]	13	86.7 ±0.8 [a]	11
	60	80.0 ±1.0 [a]	12	56.7 ±0.6 [b]	15	73.3 ±1.4 [a]	24	93.3 ±0.6 [a]	12	90.0 ±1.0 [a]	10
20	30	86.7 ±1.3 [a]	14	80.0 ±1.0 [a]	11	83.3 ±0.8 [a]	16	96.7 ±0.3 [a]	12	100.0 ±0.0 [a]	10
	60	93.3 ±0.3 [a]	12	53.3 ±1.3 [b]	14	90.0 ±0.3 [a]	20	100.0 ±0.0 [a]	11	100.0 ±0.0 [a]	10
30	30	100.0 ±0.0 [a]	14	66.7 ±0.8 [a]	14	93.3 ±1.4 [a]	17	100.0 ±0.0 [a]	12	100.0 ±0.0 [a]	11
	60	100.0 ±0.0 [a]	11	63.3 ±1.8 [a]	15	76.7 ±1.4 [b]	25	100.0 ±0.0 [a]	11	100.0 ±0.0 [a]	11
40	30	66.7 ±0.3 [a]	15	73.3 ±0.8 [a]	14	56.7 ±0.6 [b]	22	93.3 ±0.3 [a]	12	93.3 ±0.3 [a]	11
	60	77.7 ±1.8 [a]	13	33.7 ±0.3 [b]	15	70.0 ±1.7 [a]	29	96.7 ±0.6 [a]	12	93.3 ±0.6 [a]	10

Different letters indicate percentages significantly different at $p \leq 0.05$ within the sucrose concentration pair, in each species.

the calcium alginate matrix and develop shoots. For each treatment, the results were based on three replications with 10 beads per replication, with 15 beads being used in the storage treatments.

Data on regrowth were recorded and presented as means, with standard error of mean (*SEM*). Statistical analysis of percentages was carried out by a non-parametric Chi-square test ($p > 0.05$) for comparison of each pair.

Results

Effect of the polymerization time and sucrose concentration

In order to understand the influence on regeneration potential of encapsulated apical buds, different sucrose concentrations and polymerization times were applied. All results are presented in Tab. 1. An appropriate complexation time for producing beads suitable to regrowth (Fig. 2a) resulted when apical buds of *P.* × *fraseri* were hardened in the complexing solution for 30 min showing the highest level of regrowth (100%) using 30 or 60 g/L sucrose (Fig. 2b), and the shortest regrowth time (11 days) was related to the highest sucrose concentration. Hardening for 20 min showed similar results but the alginate beads were too soft and fragile.

Beads of *P. myrtifolia* showed the highest regrowth (86.7%) after 12 days, although quality of encapsulation was not satisfactory. However, the fastest regrowth (11 days) combined with adequate shoot development (80%) and an acceptable bead structure were observed when the apical buds were hardened for 20 min, adding 30 g/L sucrose (Fig. 2c,d). In this species, the highest concentration of sucrose generally determined a significant decrease of regrowth.

Encapsulated buds of *M. excelsa* showed a wide range of regrowth between 46.7 and 93.3% and the shoot development occurred within 16–29 days (Fig. 2e). In this species, among all the combinations of sucrose and polymerization times, the complexation achieved within 30 min, with an endosperm enriched of 30 g/L sucrose, gave the highest regrowth (93.3%) recorded in 17 days after sowing. Similar regrowth performance (90%) was obtained when 20 min of hardening and 60 g/L sucrose was used, but in this case, the average regrowth time was extended by up to 20 days.

In rose, the regrowth of encapsulated buds presented no significant difference between two concentrations of sucrose, in all hardening times applied. The highest regrowth percentage was recorded with 20 or 30 min polymerization times in both cultivars, independent of the sucrose concentration (Fig. 2f). The average time required for bead regrowth was shorter in 'S. Antonio' (10–11 days) than in 'Domenica' (11–13 days).

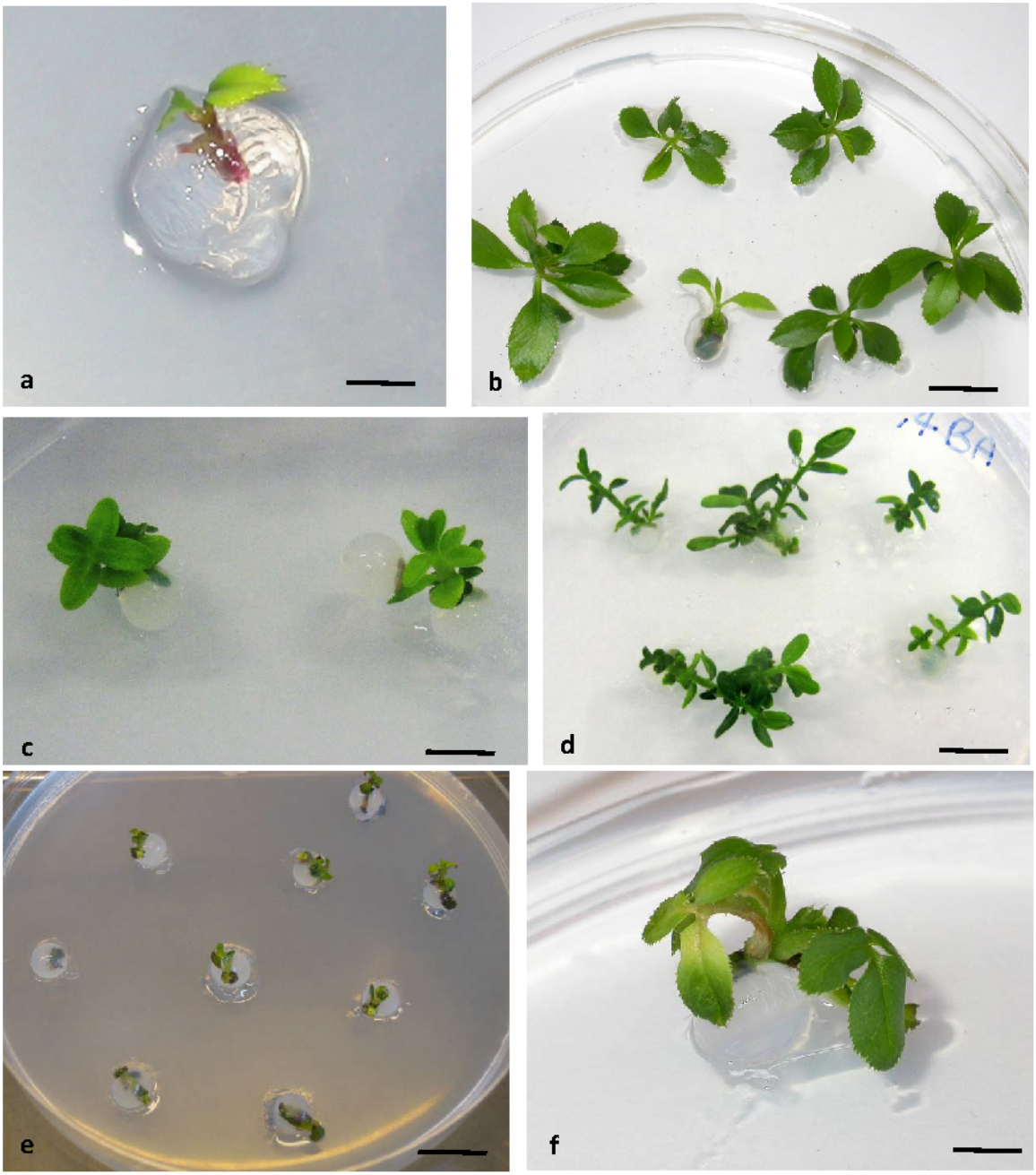

Fig. 2 **a,b** *Photinia* × *fraseri*. **a** Shoot extrusion (scale bar: 2 mm). **b** Regrowth of beads after 11 days (scale bar: 8.8 mm). **c,d** *Polygala myrtifolia*. Regrowth of beads 6 days (**c**; scale bar: 7.2 mm) and 11 days (**d**; scale bar: 13 mm) after sowing. **e** *Metrosideros excelsa*: regrowth of beads 20 days after sowing (scale bar: 12 mm). **f** Rose 'S. Antonio': regrowth of bead 11 days after sowing (scale bar: 2.6 mm).

In the present investigation, generally, the encapsulation improved both the level and the time of regrowth of explants for each species tested, comparing the results reported in Fig. 3 below relating to non-encapsulated explants.

Medium-term conservation of encapsulated apical buds

As reported above, encapsulated apical buds of *M. excelsa* and *P. myrtifolia* were stored in darkness at two temperatures (4°C and 10°C). Beads of metrosideros maintained at 10°C showed significant regrowth after 2 and 4 months of storage (93.3% and 74%, respectively) (Fig. 4), while a decline (13.3% and 6.7%) was evident in the longer conservation periods. In every case, storage at 4°C seemed to depress the regrowth of encapsulated explants, with regrowth capacity totally lost after 8 months.

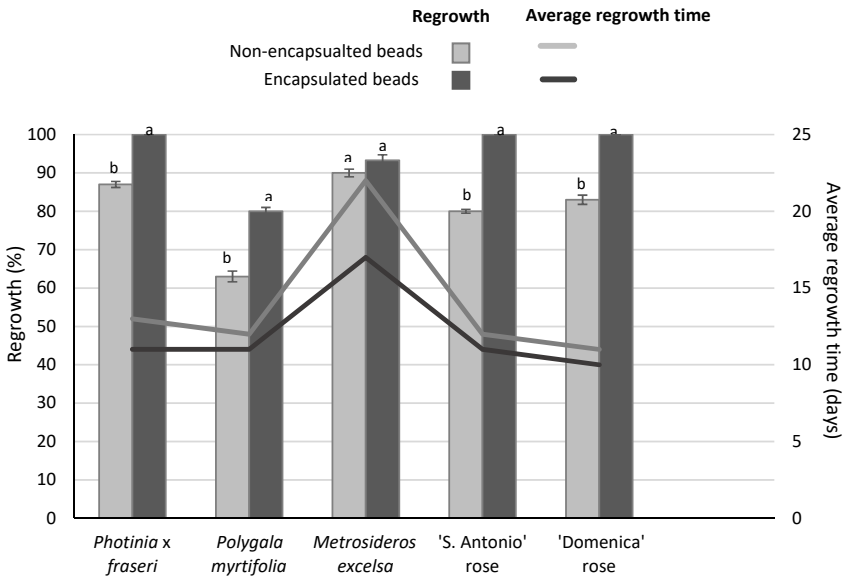

Fig. 3 Regrowth of non-encapsulated buds in standard condition in comparison with encapsulated buds obtained with the best combination (sucrose and polymerization time) in different ornamental species. Different letters indicate percentages significantly different at $p \leq 0.05$ in each species.

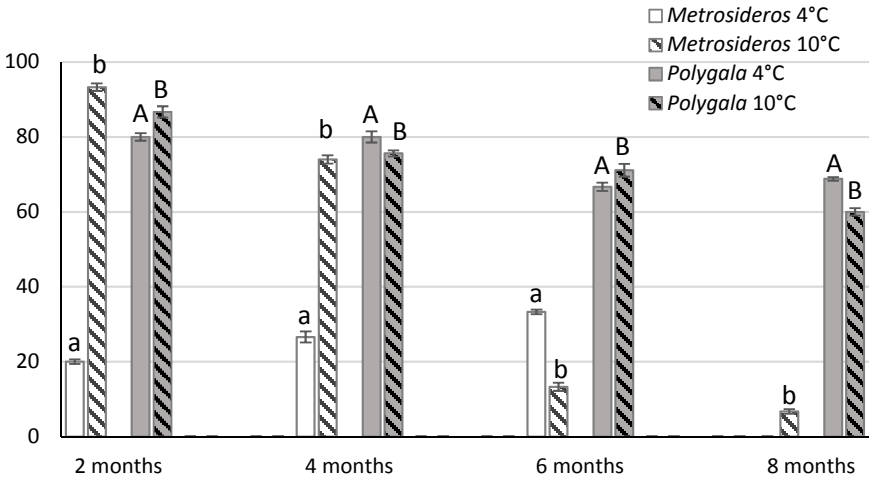

Fig. 4 Effect of temperatures (4°C and 10°C) on regrowth of *Metrosideros excelsa* and *Polygala myrtifolia* beads after different storage periods. Different letters indicate percentages significantly different at $p \leq 0.05$ within the storage temperature pair.

In contrast, beads of *P. myrtifolia* could be stored easily for 8 months, showing 68.8% (at 4°C) and 60% (at 10°C) of regrowth (Fig. 4), although a slight decrease in regrowth was observed in comparison to previous storage periods. Shoots developed from stored beads were morphologically identical to those grown under in vitro standard conditions.

Ex vitro beads recovery

In ex vitro condition, the development of *P.* × *fraseri* and *M. excelsa* encapsulated buds performed promising results with 65% and 58% regrowth, respectively, within 4 weeks of sowing (Fig. 5). However, the sprouting of buds was lower in respect to in vitro culture. Of the two species assessed, photinia responded better to the ex vitro

conditions, showing slightly higher regrowth from the second week of sowing on. In addition, more than 50% of the beads showed regrowth ability within 21 days. These preliminary results showed a limit due to explant contamination.

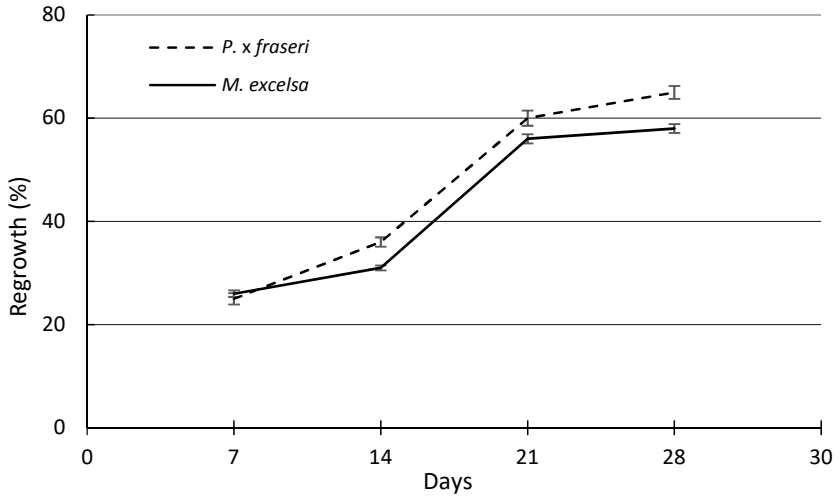

Fig. 5 Ex vitro regrowth percentage of *Photinia* × *fraseri* and *Metrosideros excelsa* beads after sowing on sterile mixture of peat and perlite.

Discussion

Effect of encapsulation on the bud regrowth

All encapsulated apical buds assessed in this study showed regrowth, with an optimal percentage and satisfactory development time compared to naked explants. In fact, the maximum regrowth in in vitro conditions was 100% in photinia and rose, and 93% and 80% in metrosideros and polygala, respectively. This could be due to the suitable composition of artificial endosperm to stimulate the regrowth of beads after sowing and it is consistent with previous literature, which reported the sodium alginate matrix provides protection and supplies nutrients [12,31].

The consistency and quality of beads are essential in effectively protecting the explants, but they are highly influenced by different concentrations of gelling matrix and complexing agent. Past research has demonstrated that 3% sodium alginate concentration is the most suitable for producing synthetic seeds of ornamental species [6,15]. Another important step in encapsulation procedure is the identification of the most appropriate time for complexation of alginate matrix, which allows proper handling without compromise the emergence of the shoot. In the present research, better-quality beads were generally achieved by complexation of a 3% sodium alginate solution with 100 mM $CaCl_2 \cdot 2H_2O$ for 20 or 30 min, depending on the species assessed. Similar reports were given for ornamental species such as *Begonia* × *hiemalis* [32], *Saintpaulia ionantha* [16], *Camellia* [33], *Vanda coerulea* [34], and chrysanthemum [18].

Besides the complexation time, in this study the effect of sugar concentration as component of the artificial endosperm was evaluated because, according to Redenbaugh [35], it represents a source of energy during the shoot extrusion and can speed up the regrowth of encapsulated propagules. The positive effect of sucrose in the gel matrix has been observed in encapsulated somatic embryos of *Solanum melongena*; the best results of germination were obtained when sucrose, either in combination with sorbitol or alone, was used as a carbon source [19]. Furthermore, in camellia, the addition into the alginate matrix of sucrose alone or added to growth regulators enhanced the germination rate of encapsulated somatic embryos [36] and sucrose was the principal determinant for the sprouting of encapsulated buds in *Betula platyphylla* [37].

Several responses were observed combining sucrose concentrations with various polymerization times. Our results showed that the optimal combination between sucrose concentration and complexation time depends on the species. When *P.* × *fraseri* buds

were encapsulated for 30 min, the addition of 60 g/L sucrose in artificial endosperm allowed explants to reach 100% regrowth, while Ozden-Tokatli and collaborators [6] reported only 86% regrowth in sucrose-free beads. This response highlighted that the shoot recovery from beads benefited of sucrose presence. Similar results were obtained in rose 'S. Antonio' and 'Domenica' cultivars, which exhibited 100% development in 11 days with a complexing time of 30 min and the addition of 60 g/L sucrose concentration. These data are consistent with Previati et al. [17], on the same species. In polygala and metrosideros, benefits were seen with the lowest sucrose concentration (30 g/L) combined with 20 min and 30 min of hardening, respectively, reaching 80% and 93% of good quality bead regrowth. The regrowth percentage recorded in metrosideros was higher in our experiment than that reported by Iapichino and collaborators [38].

In our research, the comparison between encapsulated and non-encapsulated explants confirmed the effectiveness of alginate covering in protecting explants from problems connected with the tissues' dehydration, and in providing nutritive elements essential to maintaining explant viability, especially during possible storage. In contrast, encapsulated apical buds of lilac with all sucrose concentration tested (10, 30, and 60 g/L) gave minor regrowth percentages compared to naked explants [15].

Medium-term in vitro conservation

Encapsulation technology may also be used in the short- and medium-term conservation of buds and somatic embryos of plants. The improvement of storage techniques is necessary for successful bud regrowth and synthetic seed conversion, including of elite clonal germplasm, endangered cultivars [39,40], and storage of cultivar or species in commercial laboratories. Recently, slow growth storage has found applications in in vitro conservation of several ornamental species [41].

One of the aims of in vitro plant storage is to limit the number of subcultures by reducing growth and maintaining the genetic pool in a sterile condition without compromising plant stability [42,43]. Growth rate during conservation can be limited by modifying culture medium and/or environmental conditions [44]. In vitro cold storage of plant germplasm is well documented, with the usual temperature range used being 0–5°C for temperate species, 15–25°C for tropical species. An early example of encapsulated shoot tip storage in ornamental plants was with *Camellia japonica*, where only 10% of shoots survived at 2–4°C and 7% at 18–20°C, after 75 days of conservation [45]. In the same year, Jayasree and Devi [46] reported production and regeneration of synthetic seeds in *Rosa hybrida* 'King's Ransom'; they stored the encapsulated explants up to 40 days at 4°C in the dark, achieving a survival rate of 30%.

Our results suggest that it is possible to effectively apply the encapsulation technique to extend conservation up to 8 months in the dark in metrosideros and polygala beads. For the first species, storage at 10°C was more successful, though a significant decline in regrowth during the last storage period was recorded, while at 4°C there was no regrowth at 8 months. The latter species is well suited to both temperatures tested (4°C and 10°C), demonstrating more than 60% regrowth up to 8 months of conservation.

In various ornamental species, medium-term storage was applied. Encapsulated *Syringa vulgaris* axillary buds showed a good regrowth (75%) after 50 days in darkness at 5°C [47], while *Nerium oleander* and *P.* × *fraseri* beads stored in the dark at 4°C resulted in 75% and 91% germination, respectively, after 2 or 3 months [6]. Also for *Gysophila paniculata*, 4°C was the ideal temperature to obtained conversion of encapsulated shoot tips after 90 days of storage [48]. No reduction in viability (87%) was recorded in encapsulated protocorm-like bodies of *Geodorum densiflorum* stored at 4°C for 120 days, although conservation could be continued up to 180 days with 71% of artificial seeds germinating [49].

Ex vitro beads recovery

Encapsulated propagules can be sown in ex vitro culture [3,12] using different sowing substrate [50]. In this research, recovery of photinia and metrosideros beads sown on a mixture of peat and perlite was supported by nutritive elements of artificial endosperm.

In ex vitro conditions, significant initial regrowth activity (26%) of each genotype was achieved up to 7 days from sowing. For alginate beads of photinia and metrosideros, it has been confirmed that they may be sown directly in ex vitro culture, with respective regrowth of 63% and 59% being observed.

Similar result, in the direct transfer of synthetic seeds to ex vitro conditions, has already been successfully described in bromeliads [51]. The success of ex vitro sowing depends on control of contaminations; the use of fungicides and antibiotics has already been found to prevent contamination in several species [52].

Conclusions

In conclusion, in this study, isodiametric, firm, clear, and suitable-for-handling beads were obtained, and the possibility to use in vitro-derived propagules for encapsulation of four ornamental species, as a method for improvement of regrowth and conservation, was confirmed.

In addition, the encapsulation technology gave interesting results for the medium-term preservation; indeed the beads of *Metrosideros* and *Polygala* can be well stored up to 4 months at 10°C and up to 8 months at 4°C or 10°C, respectively, without any detrimental influence on viability and plant regrowth. The results obtained are promising and useful for commercial laboratories to optimize the time and cost of large-scale production not only for tested species, but they can pave the way for other ornamental species. Moreover, the development of appropriate protocols is essential to maintain the elite disease-free clone germplasm or endangered species, as reported by various authors.

Further investigations should be carry out to reduce contamination in ex vitro condition, optimize regrowth of encapsulated buds in the soil, and promote the rooting activity.

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