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ENCAPSULATION: PROMISING TECHNOLOGY FOR NURSERIES AND PLANT TISSUE LABORATORIES

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Abstract

*The concept of encapsulation was firstly announced in 1978 by Murashige who assumed the use of this technology to protect "*vitro*-derived" somatic embryos during transport and manipulation in nurseries and farms, limiting the synthetic seed manufacture to the exclusive use of somatic embryos. At this moment, after more than 30 years of intensive research, the concept of encapsulation permits to consider that any meristematic plant tissue (obtained in* vivo *or in* vitro*) can be enclosed in a protective and nutritive matrix as long as it maintains the ability to convert into a plantlet under* in vitro *or* in vivo *environmental conditions (synthetic seed) or to evolve in shoots in a laboratory of micropropagation (capsula), also after transport and/or storage. Usually, the encapsulation procedure adopted to obtain synthetic seed or capsula includes three steps: coating, complexation and rinsing. After these steps, plant tissue portions of 3-4 mm result, enclosed in a gelling matrix which assures protection from dehydration and mechanical damages. When unipolar plant explants, as buds or microcuttings, were employed to produce synthetic seed, inductive treatment has to be applied in order to induce root emission after sowing, especially when in the encapsulation plant tissues of species with low rooting potential were involved. These treatments increase the steps to produce synthetic seed, making a large scale diffusion of the technology economically difficult for some agricultural crops. From this point of view, intense research is in progress in some laboratories, in order to look for mechanical and/or automation application, especially in the steps that require intensive manual labor. Although the encapsulation technology at the moment presents some open questions that have to be solved, interesting perspectives for its future commercial use are open because the two products of this innovative technology (capsula and synthetic seed) are useful for: the effective channel for diffusion of new plant genotypes; direct sowing of synthetic in field, greenhouse or growth chamber; plant material storage; biodiversity conservation; exchange of* in vitro *plant material between laboratories and nurseries in different countries.*

Key words: *Alginate bead, capsula, plant tissue culture, synthetic seed*

INTRODUCTION

Procedures of encapsulation were firstly hypothesized in 1978 by Murashige who assumed their possible use to protect somatic embryos regenerated by *in vitro* culture during manipulation in laboratories and transport in nurseries and farms [1]. In fact, he provided the first definition of synthetic seed (or artificial seed or synseed) as "an *encapsulated* single somatic embryo". This definition limited the synthetic seed manufacture to the exclusive use of somatic embryos *vitro*-derived and enclosed into a matrix which would allow their manipulation, transport and sowing. Afterwards, some research groups proposed the production of synthetic seeds also through the encapsulation of in *vitro*-derived propagules different from somatic embryos [2, 3, 4]. The increasing number of studies focused on the use of non-embryogenic propagules for the manufacture of synthetic seeds allowed to extend their definition as "artificially *encapsulated* somatic embryos, shoot buds or any other meristematic tissues used as functionally mimic seeds for sowing and possessing the ability to evolve into plantlets (*conversion)* under *in vitro* or *ex vitro* conditions, which can be retained even also after storage" [5, 6, 7]. So, after more than thirty years of research, the significance and the definition of the synthetic seed has clearly evolved whereas the encapsulation maintains its original and irreplaceable role for the protection of plant material, especially when *vitro*-cultured.

At this moment, this technology represents a valuable support of micropropagation (*in vitro* cloning) which has demonstrated efficiency in production of plants characterized by high genetic and sanitary qualities. For these reasons, for some decades, micropropagation has been employed in many national and international programs related to plant genetic improvement, for genotype rescue, for conservation and for exchange between laboratories and countries. Nevertheless, some problems have to be considered in relation to the commercial employment of the micropropagated plants because, after acclimatization, plantlets are cultivated in the field and face pests, pathogens, environmental and agronomic stresses, which are similarly faced by the plants produced with traditional propagation methods. In addition, the use of the micropropagated plants which are not easy to manage, store, or transport and are exposed to the deterioration and damage risks, seems to have some commercial limits in comparison with the zygotic or gamic seeds, which includes the advantages of reduced size, handling, storability and transportability, although they cannot be used for propagation of clones, especially if obtained by open pollination. In other words, micropropagated plants can lose some major advantages before commercialization, precisely when sanitary requirements have to be followed, especially when they have to overcome the problems related to the difficulties to introduce plant material in those countries which apply intransigent regulations to import plant materials [8].

Consequently, the nursery operators are looking for innovative technology able to join the advantages of micropropagation (high productive efficiency, perfect sanitary plant conditions, reduced space requirements) whit those that characterize the zygotic seed (reduced size, easier management, handling, storability and transportability). It seems that the encapsulation technology may satisfy these requirements by showing efficiency for plant material storage, conservation and facilitation in marketing and transportability.

PROCEDURE OF ENCAPSULATION

The encapsulating procedure includes three fundamental steps which are:

1. *Coating*: three–four millimeters-long explants, excised from *in vitro* or *in vivo* plant material are singly dipped into a gelling or encapsulating solution for a few seconds. For this purpose, sodium alginate is the most frequently used due to its moderate viscosity, low spin ability of solution, no toxicity to the explants, low cost and biocompatibility. Moreover, sodium alginate is employed because it provides better protection to the encapsulated explants against mechanical damages, depending on its concentration (usually ranging from 2 to 5% w/v), level of viscosity or commercial type, and from the complexation conditions. Many other substances were essayed as coating agents, in substitution to sodium alginate, like sodium alginate with gelatin, potassium alginate, polyco 2133, carboxymethyl cellulose, carrageenan, gelrite, guargum, sodium pectate, tragacanth gum [8, 9, 10].
2. *Complexation*: in order to give hardness to involucres, the alginate-coated explants are dropped into a calcium chloride solution (1.0-1.5% w/v) for 20–40 min. Ion-exchange takes place during this time, obtaining the replacement of Na+ by Ca++ forming calcium alginate [11]. When the monovalent ion of sodium is replaced by divalent ions of calcium, ionic crosslinking among the carboxylic acid groups occurs, and the polysaccharide molecules form a polymeric structure called “egg-box” [12]. Thus, the coating acquires the necessary consistence to assure protection against mechanical damages and dehydration risks. Hardening of calcium alginate bead is affected by the concentration of sodium alginate and calcium chloride and it may vary also in relation to the complexation time. Usually, higher texture corresponds to good protection during transport and manipulation, but higher difficulty to break the coating by the explant, after sowing.
3. *Rinsing*: washing the hardened involucres of explants in distilled water is required several times in order to remove the toxic residual ions of chloride and sodium. After washing, encapsulated explants can be stored before

transferring on sowing substrate to induce vegetative activity in the enclosed plant material.

The encapsulated procedure shows efficiency when the enclosed explants maintain *viability* (i.e., green colour, with no necrosis or yellowing appearance along the period between encapsulation and use), *regrowth ability* (i.e., growth of explants with consequent breakage of the involucre and extrusion of at least one small shoot or root after sowing). Usually, to achieve these conditions, nutrients and/or growth regulators were added besides the rinsing water in the solutions employed for coating and complexation. The composition of the nutritive solution is similar to that employed for the in *vitro* shoot proliferation of the micropropagation, but usually with all components at half concentration. This solution is currently called *artificial endosperm* and mimics the role of the seed endosperm because it provides nutritive support to the encapsulated explant, especially during its storage and regrowth, following the sowing [13-14].

PRODUCTS OF THE ENCAPSULATION

Other steps may be involved in the procedure of encapsulation described above, depending on the type of the explant employed and the uses of the plant material after encapsulation. In fact, by the encapsulation of plant tissue, two types of product are obtained:

a) *synthetic seed* when a whole plantlet is obtained after sowing the encapsulated explant under *in vitro* or *in vivo* conditions,

b) *capsula* which is defined as an encapsulated portion of *vitro*-derived plant tissue possessing the ability to evolve (*regrowth)* in shoot (not into a whole plantlet), and reused only inside a laboratory for micropropagation after storage and/or transport.

Redembaugh et al. [15] define the term *conversion* as the contemporary growth of epigeous and hypogeous organs, with a vascular connection between shoots and roots; this term corresponds to *germination*, which is used, as well known, for the gamic or zygotic seeds. Again, Redenbaugh [5] and Gray et al. [16] described the conversion "as the production of a green plantlet from a synthetic seed" or, in other words, the development of a whole plantlet from encapsulated somatic embryos. Subsequently, it was demonstrated that plantlets may also be obtained from unipolar encapsulated explants and, at the moment, the term conversion can be applied for all the encapsulated plant tissues able to evolve into plantlets.

ENCAPSULATION FOR CAPSULE

As reported, the *capsule* is one of the products of the encapsulation technology and it represents an effective tool for exchanging germplasm between the laboratories of different countries because they are small and relatively easy to handle [5, 17, 28, 29, 35, 36]. In fact, it is important to remember that the exchange of germplasm by the traditional methods poses severe quarantine problems because of their association with the transmission of pests and diseases. Therefore, an alternative tool for national and international plant germplasm exchange could be done by the capsula with an assurance of regrowth and proliferation abilities when subcultured again in aseptic conditions inside a laboratory of micropropagation [28, 36, 37]. The effects of the encapsulation of *vitro*-derived microcuttings or uninodal explants and the artificial endosperm composition on *in vitro* regrowth and proliferation have been examined in several plant species with good results [14, 38, 39]. The maintenance of capsules in sterile conditions and the avoidance of environmental stress (temperature and RH) during the transport are essential to maintain the viability and the regrowth abilities at high levels. Specific experiments show that transferring the capsules in small cuvettes made of semi transparent plastic material is an adequate procedure. Each locked cuvette contains 15-20 capsule and 1 mL of artificial endosperm solution is added to maintain the aseptic conditions and to avoid dehydration [40]. The capsule could also represent a supporting tool for plant material conservation, which can be carried out to allow two main goals: to lengthen the commercialization period for short- or medium-term by low temperature storage (2-8°C) and to maintain valuable genotypes for long-term by cryopreservation. As for that, Rai et al. [8] reported that the conservation of encapsulated plant explants could be achieved

through ultra-low temperature using liquid nitrogen [41, 42].

ENCAPSULATION FOR SYNTHETIC SEEDS

From nurserymen, the main expectation of the encapsulation technology is the production of synthetic seed because, as already reported, it includes the advantages of the *in vitro* propagation in terms of efficiency and quality of the propagated plants and those of the zygotic seed in term of handling, storability and transportability.

a. Synthetic seeds from somatic embryos

Structurally, somatic embryos are similar to gamic or zygotic ones and possess many useful characteristics, including the presence of both poles, epigeous and ipogeous, which assure their ability to convert into plantlets easily. Moreover, somatic embryos develop from somatic cells and this morphogenetic pathway allows clonal propagation. In any case, somatic embryos lack nutritive (endosperm) and protective (teguments or involucre) structures, which limits their application because of difficulties in handling, transport, maintenance of viability during long-term storage, and low vigor during conversion. Since by encapsulation somatic embryos are enclosed in a nutritive and protective alginate covering, this technology represents an efficient solution to overcome these problems.

In many laboratories, research groups have worked on somatic embryo encapsulation with different species including cereals, fruits, vegetables, ornamentals, medicinal plants, and forest trees; currently, efficient protocols to produce and encapsulate somatic embryos are available for some species supporting the commercial application in the nursery, especially when applied to new or valuable genotypes [8, 18]. The use of somatic embryos as encapsulating explants for synthetic seed production is limited because of the involved risks, such as: somaclonal variation, asynchronism during the somatic embryos formation and maturation, and recurrent embryogenesis [19]. However, somatic embryos are able to be produced in bioreactors by reducing their costs, making their practical use economically promising for the encapsulation technology [16]. While research is in progress in some laboratories in order to find effective solution of these problems, an interesting perspective has opened with the use of nonembryogenic (unipolar) explants obtained by *in vitro* direct organogenesis or through axillary bud proliferation. In fact, reports on encapsulated microbulbs (*Lolium longiflorum*), epiphyllous buds (*Kalanchoe tubiflora*), protocorms (*Spathoglottis plicata*, *Vanilla planifolia*, *Cymbidium giganteum* and *Dendrobium Wardianum*), rhizomes (*Nephrolepis* sp.), and hairy root fragments (*Armoracia rusticana*) have been described and their conversion into plantlet has been obtained without any particular inductive treatments [20, 21, 22]. These organs are generally easy-to obtain through direct organogenesis and risks of genetic variation are considerably reduced or absent [23, 24]. However, it is important to consider that these promising propagules can be employed for the encapsulation technology in few species, whereas currently for the synthetic seed production unipolar *in vitro* proliferated explants are largely available for many species.

b. Synthetic seeds from unipolar explants

Usually, unipolar explants used for encapsulation are nodal portions of shoots 3-4 mm-long, with apical or axillary buds, excised during or at the end of a proliferation subculture; these explants are called microcuttigs, lacking of root primordia and unable to form adventitious roots spontaneously, especially in many woody species. These characteristics represent the major problem in obtaining synthetic seed from microcuttings whereas they are suitable to obtain capsules. Instead, in some species, [14, 26], encapsulated microcuttings have demonstrated a high rooting ability after sowing, while numerous others have not performed well [8, 14, 30]. Studies conducted on plant species difficult to rooting, as apple cultivars and rootstocks, olive, kiwifruit and peach, have allowed the development of a procedure to induce rooting, in order to make the encapsulated microcuttings able to convert [7, 27, 31, 32]. This protocol, developed in the

Laboratory of *in vitro* cultures of the Department of Agricultural and Environmental Sciences (University of Perugia), considers the following six steps:

1. *Microcutting excision*: proliferated shoots are separated from proliferated clumps at the end of the subculture and the apical or axillary portions, provided with one or two buds (according to species), are used to obtain the microcuttings.
2. *Root induction*: groups of 10 microcuttings are transferred into 50-cc closed glass vessels containing 15 ml of an inductive solution made of sucrose (15 g/l) and 1-5 mg/l indole butyric acid (IBA). The cultures are kept in darkness inside the growth chamber on a 100 rpm rotary shaker for 24-72 h, according to genotype.
3. *Root primordia initiation***:** after root induction, groups of five–ten microcuttings are placed in a Magenta® vessel containing semi-solid proliferation medium, half strength, without growth regulators. The vessels are kept in the darkness, inside the growth chamber at 21 ± 2°C for 6 days.
4. *Encapsulation*: the induced microcuttings are individually used for encapsulation procedure in calcium-sodium alginate beads, enriched with the artificial endosperm, adopting the steps previously described to obtain synthetic seeds.
5. *Sowing*: after possible storage, synthetic seeds are sown *in vitro* or *in vivo* conditions, inserting them just for some millimeters into the substrate, in order to avoid asphyxia of living tissues. Agar medium or other natural substrate used for the plant cultivation can be employed as sowing supports [8, 25, 33, 34].
6. *Conversion*: usually this phase requires 4–6 weeks during which the cultures are maintained under growing conditions similar to micropropagation. After sowing of synthetic seeds, the developed plantlets are heterotrophic requiring low light intensity and trophic support; gradually they become autotrophic and adequate nutritive and environmental conditions (light and temperature) have to be assured.

PROBLEMS AND PERSPECTIVES

The research carried out on the encapsulation technology during these last three decades has permitted to obtain considerable progress. It has offered nurseries valuable tools for the production, marketing and conservation of germplasm. At this moment, large diffusion of the encapsulation technology for synthetic seed production is limited by some problems which demands effective solutions. In detail, research has to find a solution concerning:

(1) the extrusion of the encapsulated explants from the alginate coating, lowering the alginate dosage, although this implicates leaching and dehydration problems, and affects gas exchanges of the enclosed explants;
(2) the poor conversion in *vitro* and *ex vitro* conditions, studying the introduction of specific Arbuscular Mycorrhyzal Fungi (AMF) inside the synthetic seeds (*biotization*) [48];
(3) the control of fungal and bacterial contamination in non aseptic environment, verifying the effect of treatments to the sowing substrate or to synthetic seeds with specific products (fungicides, bactericides, or other antimicrobials) and the validity of biotization through AMFs and Plant Growth Promoting Bacteria (PGPB) increasing vigor and growth rate of plantlets [49, 50];
(4) the leaching of nutrients and high dehydration risks, adopting the re-inclusion of the encapsulated explants into a new alginate coating procedure (double encapsulation) or covering the alginate bead with an impermeable pellicle, although the last proposal could determine asphyxia to encapsulated propagules [51];
(5) the reduction of manual labor (i.e. cost) necessary to prepare the encapsulating explants and to carry out the encapsulation, studying automatic or semi-automatic tools and procedures. Since the mechanical excision of microcuttings suitable for encapsulation is not possible at the moment, automated procedure has been tried for synthetic seed production. In this regard, previous studies show that encapsulable adventitious shoots of M.26 apple rootstock are achievable by direct organogenesis of leaves fragments cultured *in vitro* [43] and some experiments confirm this pathway in different species [21, 44, 45]. Using this different approach, a new protocol has been proposed to automate at least some steps

involved in the production of synthetic seeds with unipolar explants. Briefly, at the end of a proliferation subculture, the hand preparation of microcuttings is substituted by the mechanical fragmentation of all clumps and the derived explants are induced to produce adventitious shoots (by direct organogenesis) which are encapsulated as *microshoots* when their size ranges from 3 to 5 mm [46, 47].

Finally, the scientific interest about the encapsulation technology and the knowledge accumulated from the research on this subject permit to suppose that, in short time, a new instrument will be available to nurseries for plant propagation, to commercialize and exchange valuable germplasm.

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