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Optimization of the conditions for production of synthetic seeds by encapsulation of axillary buds derived from minituber sprouts in potato (*Solanum tuberosum*)

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Key message: We report here, for the first time, the establishment and optimization of a method to produce synthetic seeds with axillary buds derived from potato minituber sprouts

Abstract

Synthetic seed technology is a convenient alternative to conventional multiplication in potato. In this work, we studied and optimized the process of alginate encapsulation of axillary buds derived from potato minituber sprouts. These explants, not yet used for synthetic seed production, present some advantages over other potato materials. We assayed different

concentrations of sodium alginate, $CaCl_2$ and matrix culture media, different explant sizes, different concentrations of 24-epibrassinolide applied at different stages of the encapsulation process, different planting substrates and different cold storage periods in order to determine the best conditions for encapsulation in two cultivars, ‘Santeh’ and ‘Agria’. According to our results, the conditions that resulted in the highest regrowth rates and speeds in both cultivars involved the choice of 2-3 mm-long buds, a 2 day-long pre-culture of buds in culture medium supplemented with 10^{-6}M 24-epibrassinolide, encapsulation in 3% sodium alginate with 1% $CaCl_2$ and full-strength MS culture medium, regrowth in solid MS culture medium and then transference to cocopite for conversion into plantlets. We also found that buds encapsulated under these conditions were able to retain the highest viability rate for up to 120 days in ‘Santeh’ and 90 days in ‘Agria’. although regrowth speeds decreased significantly after 60 days in both cultivars. Together, our results show that it is possible to efficiently produce synthetic seeds using axillary buds derived from potato minituber sprouts.

Keywords:

Artificial seed, alginate, cocopite, 24-epibrassinolide, germplasm storage, synseed

Introduction

In conventional potato multiplication, certified asexual propagules (commonly called *seeds* by potato growers) are produced in special seed farms, multiplied by seed growers and registered seed agencies. This system is preferable to sexual reproduction due to the genetic uniformity of the clones obtained, but it still presents some limitations, including a low multiplication rate and the progressive accumulation of degenerative viral diseases during clonal propagation. As an alternative, synthetic seed technology allows for the production of large

quantities of disease-free potato propagules from *in vitro* plantlets consuming less time and space resources (Naik and Karihaloo 2007). Additional advantages of synthetic seed production include easy handling, short and long-term storability, low production costs, facilitation of germplasm exchange between laboratories, transportation of propagules to distant places and subsequent propagation (Parveen and Shahzad 2014). The concept of the synthetic seed was first introduced by Murashige (1977), and was first demonstrated possible by Kitto and Janick (1982). At present, it is possible to produce synthetic seeds in a wide range of angiosperms, including alfalfa and celery (Redenbaugh et al. 1986), *Brassica oleracea* (Rihan et al. 2011), *Decalepis hamiltonii* L. (Sharma and Shahzad 2012), *Dendrobium* Shavin White (Bustam et al. 2012), important fruit crops such as kiwifruit (Adriani et al. 2000), apple, banana, citrus and papaya among many others (reviewed in Rai et al. 2009), and hundreds of different medicinal species (reviewed in Gantait et al. 2015).

Despite its advantages, this biotechnological approach still has some improvable aspects in order to be applied in potato at the field level. In general, the most used plant materials are somatic embryos, because they easily develop roots and shoots at the same time (Gantait et al. 2015; Redenbaugh et al. 1986). However, regeneration seems to be a major hurdle of somatic embryos compared to other explants (Gantait et al. 2015). In the particular case of potato, somatic embryogenesis is not yet commonly used, since it highly depends on the genotype and explant used and in general, the rates of somatic embryo induction are still low (Nassar et al. 2015). In addition to somatic embryos, other explants such as shoots and nodes with apical or axillary buds have been used in synthetic seed technology (reviewed in Gantait et al. 2015). Compared with somatic embryos, non-embryogenic micropropagules are cheaper to produce and easier to handle. This is why the possibilities of encapsulating alternative materials such as potato nodal segments (Sarkar and Naik 1998, 1997), shoot tips (Nyende et al. 2003) and

77 cell suspension cultures (Schafer-Menuhr et al. 2003) were soon explored. In this work, we
78 explore the use of axillary buds derived from potato minituber sprouts (PMS) for
79 encapsulation in synthetic seeds.
80
81 One of the principal factors for a successful production of synthetic seeds is to find an
82 encapsulation material consistent enough to allow for seed handle without breakage, but weak
83 enough to allow for the bud to emerge from the capsule upon regrowth inititation
84 (Redenbaugh et al. 1986). This delicate balance between hardness and weakness can be
85 achieved encapsulating with alginate hydrogels, which is by far the most used substance for
86 explant encapsulation (Rai et al. 2009; Gantait et al. 2015). Synthetic seeds must also contain
87 a matrix with enough nutrients to ensure seed-to-plant development, and in some cases,
88 growth regulators to control explant regrowth (Sharma and Shahzad 2012). Brassinosteroids
89 are a class of growth regulators known to influence a range of growth and developmental
90 processes both *in vivo* and *in vitro*. Among the tens of different types of brassinosteroids
91 identified, 24-epibrassinolide (EBr) is one of the most active when applied exogenously
92 (Fujioka and Yokota 2003). In this context, we studied the role that brassinosteroids might
93 have in the process of synthetic seed production, when applied at different concentrations and
94 at different stages of explant preparation and encapsulation. We also studied other parameters
95 of interest such as the optimal explant size, the effect of cold storage in regrowth potential,
96 and the influence of using different substrates for bud-to-plantlet conversion. Our study was
97 performed in parallel in two potato cultivars, ‘Santeh’ and ‘Agria’. Altogether, our results
98 allowed for the establishment and optimization of a method to produce synthetic seeds using
99 PMS-derived axillary buds.
100
101 **Material and Methods**

Plant material and explant preparation

We used minitubers of two potato cultivars, Santeh and Agria, supplied by the Agriculture and Natural Resources Research Center of Hamedan, Hamedan, Iran. Before use, potato minitubers were washed under running water for 30 min, treated with 1 g/l carbendazim for 10 min to remove fungi, then surface sterilized by immersion in 70% ethanol for 30s and 5% sodium hypochlorite for 20 min, and finally washed with sterile distilled water three times (4 min each). In order to form PMS, minitubers were kept for 2 months at 16/8h photoperiod and 21/18°C (day/night) temperature under 40 $\mu mol.m^{-}2.s^{-1}$ light, PMSs were formed after 15 days in cv. 'Santeh' and after 21 days in 'Agria'. Axillary buds (approximately 2–3 mm in size) were excised under a binocular microscope from 2 month-old PMSs (15 mm in size) and used as explants for encapsulation. These explants were pre-cultured for 2 days in solid MS medium (Murashige and Skoog 1962) pH 5.7, supplemented with 10 mg/l naphthalene acetic acid (NAA), 30 g/l sucrose and 8 g/l agar.

Explant encapsulation

To produce synthetic seeds, we first prepared autoclaved solutions of sodium alginate and $CaCl_2$ at different concentrations as described below. Droplets of the alginate solution, each containing one pre-cultured axillary bud (Fig. 1A), were poured with a 5 mm-wide sterile plastic pipette into 100 ml matrix culture medium, supplemented with $CaCl_2$. For all experiments, matrix culture medium consisted of MS medium (pH 5.7) with 5 mg/l benzyladenine (BA), 10 mg/l NAA and 300 mg/l activated charcoal. Bud-containing droplets were kept in a $CaCl_2$ solution (pH 5.7) for 25 min under continuous shaking on a magnetic stirrer until complete crosslinking of sodium alginate. Then, alginate capsules (~5–7 mm, Fig. 1B) were collected, thoroughly washed thrice with sterile distilled water to remove traces of

$CaCl_2$, and subjected to a cold treatment in Petri dishes at 4°C for 2 days. Encapsulated buds were then placed in regrowth medium consisting of solid MS medium (pH 5.7) with 30 g/l sucrose and 8 g/l agar, but without growth regulators. After two weeks at 25ºC and 100 $\mu mol.m^{-2}.s^{-1}$ (16/8 photoperiod), regrowth (stem elongation, shoot and then root development; Figs. 1C, D) was evaluated in terms of regrowth rate and speed. Regrowth rate was defined as the percentage of encapsulated buds that elongated and emerged out of the capsule wall to produce normally-growing *in vitro* plantlets. Regrowth speed was calculated according to Maguire's equation (Maguire 1962): M = n1/t1 + n2/t2 + … + nn/tn, where n1, n2, … nn are the number of emerged buds at times t_1, t_2, …, t_n measured in days.

Experiment I: effect of different combinations of sodium alginate and $CaCl_2$

In this experiment, we assessed the effect in regrowth rate and speed of different concentrations of sodium alginate (2.5, 3.0, and 3.5% w/v) in matrix culture medium. For crosslinking, two concentrations of $CaCl_2$ (1.0 and 1.5%, w/v) were tested.

Experiment II: effect of explant bud size and matrix culture medium

To assess the effect of bud size and matrix culture medium in regrowth rate and speed, we prepared axillary buds of two different sizes (1-2 mm and 2-3 mm). These buds were encapsulated in half and full strength MS culture medium, keeping unchanged the rest of components of the matrix culture medium.

Experiment III: effect of EBr concentration and stage of application

In this experiment, encapsulated 2–3 mm axillary buds from cvs ‘Santeh’ and ‘Agria’ were treated with different concentrations of EBr: 0, 10^{-6}M, 10^{-7}M and 10^{-8}M. We also tested the application of EBr at three different stages: during pre-culture of axillary buds for two days

(S1), in the matrix culture medium (S2), and during culture of encapsulated buds in regrowth medium for 2 days (S3). In addition to regrowth rate and speed, in this experiment we also measured the length of elongated shoots to evaluate the effects of EBr.

Experiment IV: effect of planting substrate

In this experiment we assessed the effect of four different substrates in the promotion of regrowth of encapsulated axillary buds. These substrates included MS culture medium without growth regulators (Fig. 1D) and pots containing cocopite (Fig. 1E), perlite and soil mixture. To assess this, we first evaluated the regrowth rate and speed of each substrate. Second, we evaluated the suitability of each substrate in the conversion of encapsulated buds into plantlets (Fig. 1F) after four weeks. For this, we cultured synthetic seeds in basal MS medium for two weeks, and then transferred growing buds to each of the substrates studied. After four weeks, we measured in regenerated plantlets growth traits such as root and shoot length, stem diameter and number of leaves. For all the assays of this experiment, synthetic seeds were covered with polyethylene sheets until shoot and root emergence (7-14 days), in order to maintain a high humidity level. As a control, we used non-encapsulated axillary buds placed in the same substrates and kept always under similar conditions.

Experiment V: effect of storage at low temperature

In our final experiment, we evaluated the regrowth potential of encapsulated buds after different periods of storage at low temperature (0, 60, 90 and 120 days). Encapsulated buds were stored at 4ºC in culture dishes with or without solid MS medium. After each storage period, encapsulated buds were transferred to fresh basal MS medium for conversion into plantlets. Regrowth rate and speed were measured after 2 weeks of culture.

Data analysis

For each experiment, 3 replicates were performed. The experimental unit was six synthetic seeds per culture dish. Data of all experiments were subjected to analysis of variance (ANOVA) using a factorial design. Mean separations were carried out using Duncan's multiple range test (Duncan 1955) with $p \leq 0.05$. Percentage data were subjected to square root transformation before analysis. SPSS 16 software was used for data analysis.

Results

Evaluation of the encapsulation conditions

In this experiment, PMS-derived axillary buds (2-3mm in size, Fig. 1A) were encapsulated using different concentrations of sodium alginate (2.5%–3.5%) and $CaCl_2$ (1.0% and 1.5%). Each combination of sodium alginate and $CaCl_2$ produced synthetic seeds with different shapes, textures, firmness and transparency. The highest regrowth speed was obtained combining 2.5% sodium alginate with 1% $CaCl_2$. However, 2.5% sodium alginate produced fragile capsules, prone to breakage and difficult to handle. The combination of 3% sodium alginate with either 1% or 1.5% $CaCl_2$ produced the best overall performance, with good results in terms of regrowth rates (Table 1) and hydrogel formation, producing firm, clear and isodiametric capsules (Fig. 1B), easy to break as soon as the bud initiates regrowth (Fig. 1C). Alginate concentrations above 3% produced isodiametric capsules too, but they were too hard and caused a considerable delay in germination, as revealed by the reduced regrowth speeds. Next, we evaluated the influence of explant size and concentration of MS salts in the matrix culture medium. For both cultivars, the use of larger (2-3 mm) buds yielded better results, in terms of regrowth rate and speed, than using small (1-2 mm) buds (Table 2). We found that in

general, the values of regrowth rate and speed were slightly higher for full strength MS (Table 3). However, these differences were only significative in the case of large buds of cv 'Santeh'. In other words, results were similar. Thus, we concluded that the best conditions to optimize bud regrowth would be to excise 2-3 mm buds, and to embed them in 3% sodium alginate with MS medium, and 1% $CaCl_2$.

Effect of EBr concentration and stage of application

In this experiment, we evaluated the effect in regrowth of adding different concentrations of EBr at different stages of the process (S1, S2 and S3). Table 4 shows the results obtained in cv 'Santeh'. Analysis of these results clearly evidenced that the best concentration of EBr is 10^{-6}M. When applied at S1, this concentration gave the best results for the three parameters studied: regrowth rate, speed, and shoot length. When applied at S2 or S3, no significant differences among concentrations (including control with no EBr) were found in any of the three parameters measured, which indicates that EBr can be omitted at S2 and S3. Results in 'Agria' (Supplementary Table S1) confirmed that the best concentration of EBr is 10^{-6}M. This concentration yielded the best results for the three parameters studied when applied at S1. In contrast to 'Santeh', application of 10^{-6}M EBr at S2 in 'Agria' was also beneficial. However, the results at this stage were not different from those of S1. Therefore, there was no clear improvement in 'Agria' with the application of EBr at S2, with respect to S1. At S3, no differences at all were found among control and EBr-treated samples. In summary, we concluded that the addition of 10^{-6}M EBr during pre-culture of explants in MS medium, prior to encapsulation (S1), is beneficial for further bud regrowth in the two cultivars checked.

Effect of planting substrate

The substrate used for planting showed a strong influence in regrowth of synthetic seeds, being this influence genotype-dependent. In general, 'Santeh' responded better than 'Agria', as shown in previous experiments. For both cultivars, the best results in terms of regrowth rate and speed were obtained with the use of MS medium without growth regulators (Fig. 1D), which markedly outperformed the results of commercial substrates (Table 5). Among them, the best results were obtained with the use of cocopite (Fig. 1E)..These positive results of cocopite in regrowth were consistent with its effects in conversion of growing buds into plantlets (Fig. 1F) after four weeks. Table 6 shows that cocopite produced significantly longer roots and shoots, wider stems and more leaves than perlite and soil mixture, for both 'Santeh' and 'Agria' cultivars. Therefore, the best results for bud regrowth and conversion to plantlets would be obtained by incubating synthetic seeds in basal MS medium for two weeks and then planting in cocopite.

Effect of storage at low temperature

We stored synthetic seeds of 'Santeh' and 'Agria' at 4ºC during 0, 60, 90 and 120 days, either alone or in solid MS culture medium. For both cultivars, the results obtained at each time point with the two methods assayed were not significantly different, neither in terms of regrowth rate nor in terms of speed. For this reason, we considered the results of both storage methods together. As expected, 'Santeh' showed higher regrowth rates and speeds than 'Agria' at all time points. In 'Santeh', regrowth rates were not affected by prolonged storage at 4ºC compared with controls (0 day value) without storage (Fig. 2A). In 'Agria', only the 120-day stage produced values clearly lower than the rest. In contrast, regrowth speed was more affected by prolonged cold storage. Both cultivars showed a significant decrease after

90 days (Fig. 2B). In both cultivars, non-encapsulated buds lost their viability two days after excision from PMSs. Therefore, cold storage of these explants without encapsulation was not possible.

Discussion

In this study, we showed that PMS-derived axillary buds encapsulated in alginate–MS are suitable for potato clonal propagation. Although there are other explants that have been traditionally used to produce synthetic potato seeds, we think that PMS-derived axillary buds have additional advantages that make them an ideal explant to encapsulate. The first is efficiency, since a lot of axillary buds (around 30-80) can be obtained from a single minituber. The second is the high viability of the explants after several months of cold storage, as shown in this work. The third is the genetic stability of axillary buds. A phenotypic, cytogenetic and molecular comparison among four different ways to propagate potato (somatic embryos, axillary bud proliferated shoots, microtubers and true potato seeds), revealed that potato axillary buds are the most genetically stable propagules, at least in terms of AFLP polymorphisms (Sharma et al. 2007). Indeed, axillary branching is thought to present the lowest risk of generating genetic instability among the different alternatives for clonal propagation (Shenoy and Vasil 1992). For these reasons, we think that the method described hereby is a simple, stable, quick, highly cost-effective and therefore convenient method for potato propagation in a reduced space.

We also optimized the conditions for bud encapsulation, regrowth storage, and planting. The principal factors affecting encapsulation and regrowth include the concentration of sodium alginate, $CaCl_2$ and MS salts used for bud preparation, the size of the explants used, and the

277 presence of growth regulators during this process. We found that the best way to produce
278 firm, clear, isodiametric and easy-to-handle capsules is to use 3% sodium alginate and 1 or
279 1.5% $CaCl_2$ for gelling. The convenience of 3% sodium alginate for producing hard and well-
280 shaped capsules has been previously reported in potato nodal segments (Sarkar and Naik
281 1997) and in shoot tips and nodal segments of *Corymbia torelliana* × *C. citriodora* and *Khaya*
282 *senegalensis* (Hung and Trueman 2012a, b). Our study adds to those pointing out that low
283 concentrations of sodium alginate (2.5% or lower) do not solidify well, producing fragile
284 capsules, difficult to handle during transference (Larkin et al. 1988; Sharma and Shahzad
285 2012). In turn, high concentrations (higher than 3%) would give rise to excessively hard
286 capsules, difficult to break up and therefore promoting a considerable delay in bud regrowth
287 (Sharma and Shahzad 2012). Another parameter potentially affecting capsule properties is the
288 concentration of MS salts in the matrix culture medium. Good results (76–100% shoot
289 regrowth) have been previously obtained in this and other species with full strength MS as
290 well as with half strength MS (Sarkar and Naik 1998, 1997; Nyende et al. 2003; Hung and
291 Trueman 2012a), In our hands, we found no benefit in using half strength MS. Explant size
292 seems also to influence regrowth. Previous studies dealing with the production of synthetic
293 seeds from protocorm-like bodies in *Dendrobium* Shavin White suggested that larger explant
294 sizes produce higher regrowth rates (Bustam et al. 2012). In potato axillary buds from PMS,
295 we showed that this holds also true, since 2-3 mm bud sizes allowed for the regrowth of more
296 explants, and for a faster regrowth.
297
298 We also improved regrowth of encapsulated buds by studying the effect of EBr application at
299 different stages. Brassinosteroids are growth regulators first discovered in pollen of *Brassica*
300 *napus* (Grove et al. 1979), but now found in many different species, from algae to
301 angiosperms. They are present in most organs of the plant, and even at very low

concentrations, brassinosteroids have been shown to promote cell growth, differentiation and elongation (Brosa 1999), as well as protection against biotic and abiotic stresses. This is why they have been widely used to promote proliferation and differentiation in different *in vitro* processes including adventitious shoot regeneration (Sasaki 2002), callus formation and plant regeneration (Nuñez et al. 2004), microspore embryogenesis (Corral-Martínez and Seguí-Simarro 2014), somatic embryogenesis (Pullman et al. 2003; Azpeitia et al. 2003), or protoplast culture (Oh and Clouse 1998), among others. However, our study revealed that 10^{-6}M EBr application is most beneficial when applied not during bud regrowth but during explant pre-culture, prior to encapsulation and further regrowth. This made us think that perhaps, the EBr role in cultured axillary buds would be more related to a protective effect against biotic and abiotic stresses. Indeed, it is known that EBr exerts anti-stress effects, both independently as well as through interactions with other growth factors (Divi et al. 2010). EBr has been reported to regulate the activities of antioxidative enzymes and antioxidants, protecting the plants under different biotic (Nakashita et al. 2003) and abiotic stresses, including drought, salinity, heat and cold stresses (Kagale et al. 2007). It is important to note that, as a part of the encapsulation procedure, buds wer subjected to a cold treatment at 4°C for 2 days. It is tempting to speculate that EBr might contribute to protect PMS-derived buds against this cold temperature.

In this work we also tested different conditions for bud regrowth and conversion to plantlets, finding that the most efficient combination is to incubate synthetic seeds in basal MS medium and then planting the regrowing buds in cocopite. These results suggest that the conversion rate and viability of synthetic seeds somehow depend on the physical structure of the culture substrates used. According to previous reports, it seems that synthetic seeds of different species would require different substrates. For example, vermiculite, sand and soil would be

suitable for mulberry (Machii and Yamanouchi 1993), perlite for M.26 apple rootstock (Micheli et al. 2002), and perlite and compost for cauliflower (Rihan et al. 2011). It appears clear that optimal conditions must be determined for each species.

Another important parameter to study is the storage time without loss of viability. Considering that non-encapsulated axillary buds lost their viability two days after excision from PMSs, we found that using our procedure, synthetic seeds can be stored at 4ºC for up to 90 days without significant viability loss. For 'Santeh', this time was extended to 120 days, indicating that for certain cultivars, it could even be longer. As suggested by Sharma et al. (2014), the viability decrease could be attributed to an inhibition of tissue respiration due to the difficulties for oxygen diffusion imposed by the alginate matrix, or to a loss of moisture due to partial desiccation during storage. Our observations are in line with or even better than the viability after cold storage reported for many other species (Faisal and Anis 2007; Ahmad and Anis 2010). Future directions should focus on the extension of the storage period without viability loss, as achieved for example in *Cineraria maritima*, where synthetic seeds can be stored up to six months keeping a regrowth rate of 82.35% (Srivastava et al. 2009).

In summary, we described for the first time and optimized a procedure for encapsulation of PMS-derived axillary buds by selecting the best explant size, encapsulation in 3% sodium alginate with 1% $CaCl_2$ and MS basal medium as matrix. Supplementation of pre-culture medium with 10^{-6}M EBr improved the regrowth properties of potato synthetic seeds. Cocopite was the best commercial substrate for regrowth and conversion of encapsulated axillary buds into plantlets. This study also determined the maximum span of cold storage without viability loss in 'Santeh' and 'Agria'. This study establishes the conditions for short-mid term storage

of encapsulated axillary buds and opens the door for further refinements of this alternative for potato synthetic seed production.

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465

Tables

Table 1. Effect of different concentrations of sodium alginate and **$CaCl_2$** on regrowth rate and speed of encapsulated buds from 'Santeh' PMSs after 2 weeks of culture. Different letters indicate significant differences according to Duncan's multiple test.

Sodium alginate (%)	$CaCl_2$ (%)	Regrowth	
		Rate	Speed
2.5	1	55.55 ab	0.76 a
	1.5	44.44 bc	0.24 c
3	1	61.11 a	0.46 b
	1.5	55.55 ab	0.48 b
3.5	1	11.11 d	0.06 d
	1.5	33.33 c	0.28 c

Table 2. Effect of explant size on regrowth rate and speed of encapsulated buds after 2 weeks of culture. Different letters indicate significant differences according to Duncan's multiple test.

Cultivar	Bud size (mm)	Regrowth	
		Rate	Speed
Santeh	1-2	33.33 b	0.25 bc
	2-3	77.77 a	1.04 a
Agria	1-2	19.44 c	0.14 c
	2-3	38.89 b	0.34 b

Table 3. Effect of the concentration (strength) of MS salts on regrowth rate and speed of encapsulated buds after 2 weeks of culture. Different letters indicate significant differences according to Duncan's multiple test.

Bud size (mm)	Cultivar	MS medium strength	Regrowth rate	Regrowth speed
1-2	Santeh	Full	38.89 c	0.32 bc
		Half	27.77 cd	0.18 bc
	Agria	Full	22.22 cd	0.17 bc
		Half	16.66 d	0.11 c
2-3	Santeh	Full	88.89 a	1.13 a
		Half	66.66 b	0.95 a
	Agria	Full	38.89 c	0.30 bc
		Half	38.89 c	0.38 b

Table 4. Effect of the stage of application of EBr in regrowth rate, speed and shoot length of encapsulated buds of cv 'Santeh' after two weeks of culture. See text for details on the S1, S2

and S3 stages. Different letters indicate significant differences according to Duncan's multiple test.

Stage	EBr concentration (M)	Regrowth rate	Regrowth speed	Shoot length (cm)
S1	0	77.77 b	1.11 bc	4.63 bc
	10^{-6}	100.00 a	1.40 a	4.87 a
	10^{-7}	88.89 ab	1.16 bc	4.57 c
	10^{-8}	83.33 ab	1.01c	4.61 c
S2	0	83.33 ab	1.14 bc	4.66 bc
	10^{-6}	94.44 ab	1.29 ab	4.77 ab
	10^{-7}	88.89 ab	1.11 bc	4.65 bc
	10^{-8}	83.33 ab	0.99c	4.55 c
S3	0	77.77 b	1.09 bc	4.62 c
	10^{-6}	83.33 ab	1.13 bc	4.68 bc
	10^{-7}	88.89 ab	1.14 bc	4.64 bc
	10^{-8}	88.89 ab	1.14 bc	4.67 bc

Table 5. Effect of different substrates in regrowth rate and speed of encapsulated axillary buds after 2 weeks of culture. Different letters indicate significant differences according to Duncan's multiple test.

Cultivar	Substrate	Regrowth rate	Regrowth speed
Santeh	MS	94.44 a	1.40 a
	Cocopite	61.11 b	0.41 c
	Perlite	44.44 cd	0.24 d
	Soil mixture	27.77 ef	0.13 ef
Agria	MS	50.00 bc	0.64 b
	Cocopite	33.33 de	0.26 d
	Perlite	27.77 ef	0.16 e
	Soil mixture	16.67 f	0.07 f

Table 6. Effect of different substrates in the conversion of growing buds into plantlets after four weeks. Different letters indicate significant differences according to Duncan's multiple test.

Cultivar	Substrate	Root length (cm)	Shoot length (cm)	Stem diameter (mm)	Number of leaves
Santeh	Cocopite	11.67 a	7.67 a	2.06 a	4.67 a
	Perlite	9.67 b	5.33 b	1.63 b	3.33 b
	Soil mixture	6.67 c	4.33 c	1.43 cd	2.33 c
Agria	Cocopite	6.17 c	4.17 c	1.50 c	3.67 ab
	Perlite	5.33 d	3.17 d	1.36 d	2.33 c
	Soil mixture	3.17 e	2.57 e	1.21 e	1.00 d

493 **Figure legends**

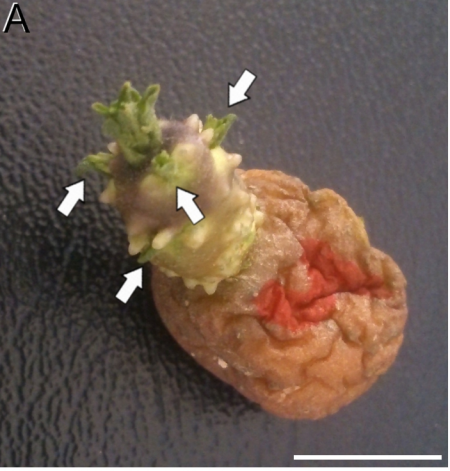
494

495 **Fig. 1.** Production of synthetic seeds with axillary buds from potato minituber sprouts. A:
496 potato minitubers showing axillary buds (arrows). B: axillary buds encapsulated in calcium
497 alginate beads. C: Shoot and root emergence from encapsulated buds. D: Shoot regrowth and
498 elongation. E: Conversion of regrowing buds into plantlets in cocopite-containing pots. F:
499 Acclimated plantlet obtained from encapsulated axillary buds after 4 weeks of culture.

500

501 **Fig. 2.** Effect of different storage times at 4°C in regrowth rate (A) and regrowth speed (B) of
502 encapsulated axillary buds derived from 'Santeh' and 'Agria' PMSs.

503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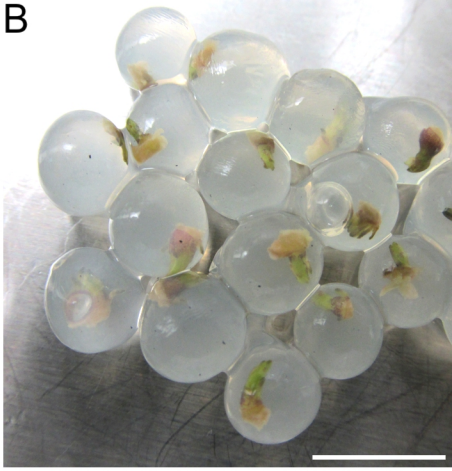
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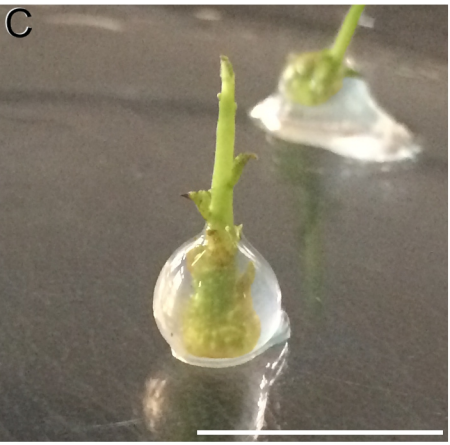
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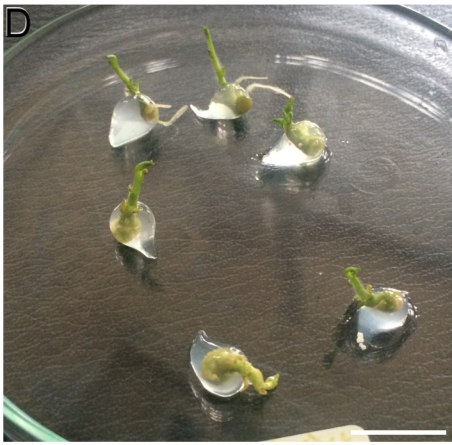
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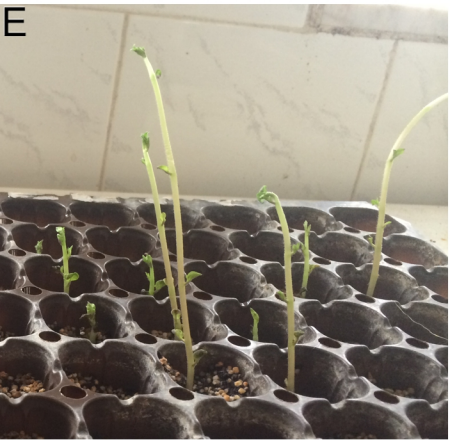
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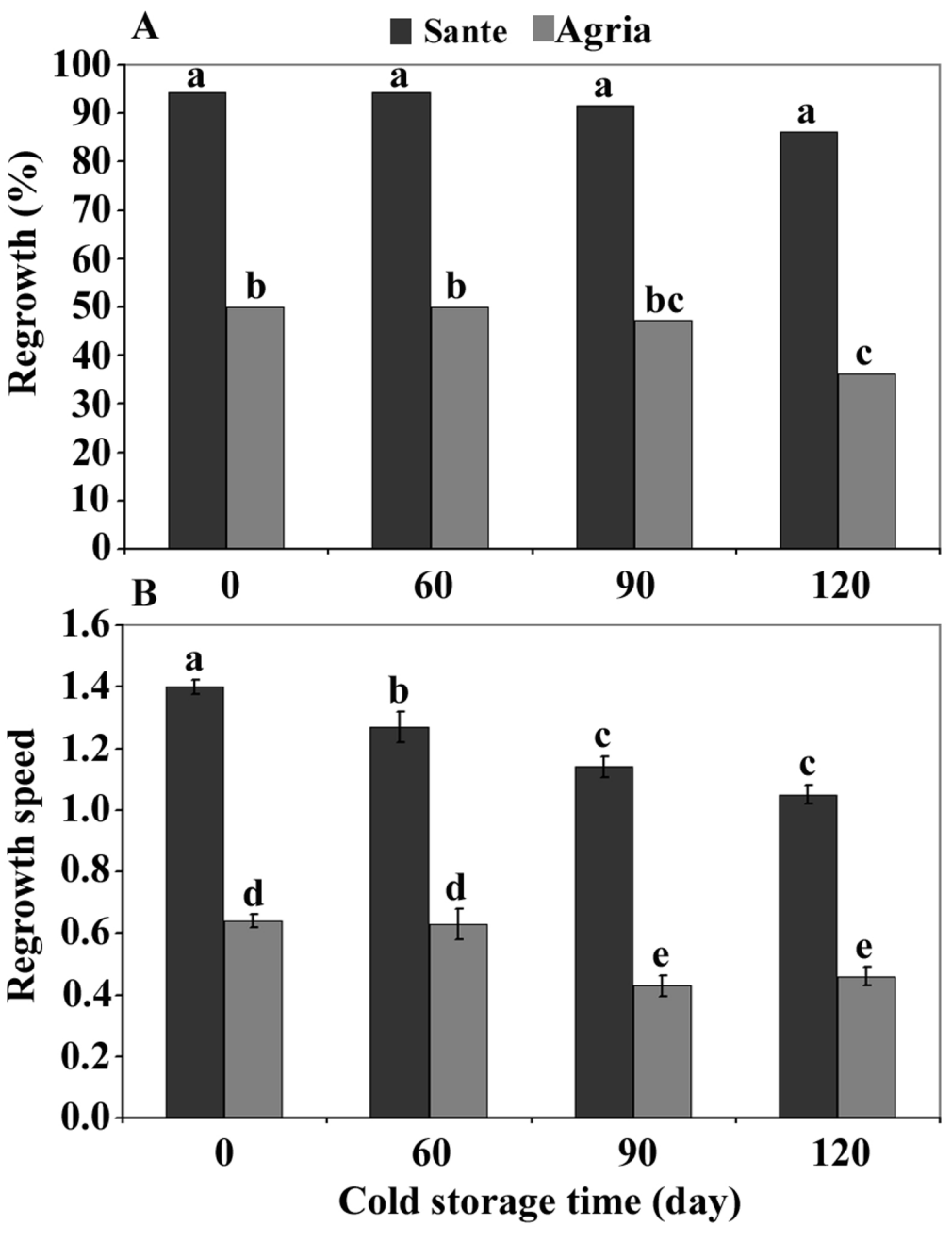
A
Sante
Agria
Regrowth (%)
100
90
80
70
60
50
40
30
20
10
0
a
a
a
a
b
b
bc
c
0
60
90
120
B
Regrowth speed
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
b
c
c
d
d
e
e
0
60
90
120
Cold storage time (day)