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RESEARCH ON THE USE OF SYNTHETIC SEED IN CONSERVATION OF *KALANCHOE BLOSSFELDIANA*

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Abstract

Synthetic seed technique can be a useful tool in plant conservation, as it combines the advantages of vegetative and generative propagation: uniformity of the material, lower costs for storage and transportation and the production of pathogen-free planting material. Kalanchoe blossfeldiana in vitro derived explants were used to examine the influence of sucrose concentration in the encapsulation maxtrix and the influence of salt concentration in the storage medium on the conservation of this species. Nodal segments and shoot tips were encapsulated in alginate solution containing two different sucrose concentrations (2% and 4%) and stored for 7,30 and 60 days in liquid medium with different MS salt concentrations (normal, halved and quartered). Statistical tests did not reveal an influence of the sucrose concentration in the alginate solution. On the other hand, tests indicated that the variants with the low salt concentrations (halved and quartered) showed better growth in terms of average shoot length and average number of leaves.

Key words*: artificial seed, Kalanchoe blossfeldiana, micropropagation, plant conservation, synthetic seed.*

INTRODUCTION

Kalanchoe blossfeldiana is one of the most economically important ornamental pot plants grown worldwide. *Kalanchoe blossfeldiana* originally has 2n=34 chromosomes, but modern varieties obtained by inter-specific hybridization have higher levels of ploidy (2n=51 triploid, 2n=68, tetraploid) (Van Voorst and Arends, 1982). Seed propagation was a common propagation method for early cultivars, but vegetative propagation is commonly used nowadays because it offers uniformity in plant growth, growing habit and flower colour (Sanikhani et al., 2006). *In vitro* propagation of *Kalanchoe blossfeldiana* was achieved by numerous authors (Dickens & Staden, 1990; Ioannou & Ioannou, 1992; Sanikhani et al., 2006; Frello et al., 2002; Kaviani et al., 2014; Kale et al., 2018).

Synthetic seeds are represented by somatic embryos, axillary or terminal buds, nodal segments, cell aggregates or other types of artificially encapsulated tissues that can be used for sowing and have the ability to transform into plants, and that can retain this ability even after short term and medium term storage (Hussain et al., 2000; Micheli & Standardi, 2016; Magray et al., 2017).

The concept of synthetic seed was first introduced in 1977, by Murashige. Initially, the term referred to encapsulated somatic embryos (Murashige, 1977), but later, the concept was extended to non-embryogenic tissues as well. (Bapat et al., 1987). Synthetic seeds were created as a means to make the somatic embryos more similar to zygotic ones, by creating a protective layer aiming to make manipulation and storage easier, so that they can be used in propagation and germplasm conservation (Magray et al., 2017). The use of somatic embryos has been studied on several plant species, by numerous authors: *Rotula aquatica* (Chithra et al., 2005), *Oryza sativa* (Kumar et al., 2005), *Pinus radiata* (Aquea et al., 2008), *Quercus suber* (Pintos et al., 2008), *Litchi chinensis* (Das et al., 2016). The advantage of using somatic embryos over other types of tissue for encapsulation, is their bipolar structure, thus their ability to regenerate roots and shoots simultaneously, without any specific treatment (Hussain et al., 2000). However, a main disadvantage of so-

matic embryos is that their structures do not possess an endosperm and protective tissues, and they are dependent on the culture media and their manipulation and storage is difficult (Hussain et al., 2000; Magray et al., 2017; Lambardi et al., 2006).

Because of the limitations that somatic embryos possess (asynchronous development, lack of protective tissues, difficulty of creating a protocol for each species), in 1987, Bapat proposed the use of non-embryogenic tissues to make synthetic seeds, especially in species that are recalcitrant to somatic embryogenesis. Generally, those type of explants (shoot tips, nodal segments) are easier to obtain compared to somatic embryos, the risk of somaclonal variations is reduced and can be applied to most species (Standardi and Micheli, 2013).

Regarding the technology used, there are two types of synthetic seeds: dehydrated and hydrated. Dehydrated synthetic seeds are produced from somatic embryos, either not encapsulated or encapsulated in polyethylene glycol, and then dehydrated. Dehydrating the somatic embryos increases their storage period and survival rate, but this technology can only be applied to embryos that tolerate the dehydration process (Magray et al., 2017). Hydrated synthetic seeds designates/ represents the encapsulation of explants (somatic embryos, shoot tips, nodal segments, callus) in different hydrogel solutions: sodium alginate, potassium alginate, sodium pectate, carrageenan, gelrite (Hussain et al., 2000).

The technology of synthetic seeds has many applications: it can be used to asexually propagate endangered species, and valuable genotypes of species that normally don't produce seeds (Qahtan et al., 2019). The technique can also be used for exchange between laboratories and institutions and for short and medium conservation of germplasm (Standardi and Micheli, 2013).

Long term conservation of synthetic seeds can be achieved by cryopreservating the plant material. Otherwise, these can be stored on short and medium time in the fridge, at 2-8°C , depending on the species (Micheli & Standardi, 2016). Synthetic seeds can be stored up to 90 days in the fridge, but the optimum storage period is dependent on the species, but generally, most species cand be stored in the fridge, at 4-6°C (Qahtan et al., 2019). Some species can be stored at room temperature, in dark conditions (Standardi & Micheli, 2013). High humidity and low temperature are essential for storing the synthetic seeds (Mallikarjuna et al., 2016).

The advantage of synthetic seeds is that it combines the advantage of asexual propagation with the seed propagation: easy storage, easy transportation, the possibility of using sowing machinery, protection against pests and disease, the production of virus free planting material (Lambardi et al., 2006).

Plants developed from synthetic seeds are phenotypically identical with the plant that was used as the explant source. Synthetic seeds can be produced in a short period of time (one month), compared with natural seeds, which are the result of a complex reproductive process and that extends over a longer period of time. Also, they can be produced in any season of the year, as they production is not dependent on season and on field conditions. Theoretically, synthetic seeds don't require the acclimatization phase that is mandatory for *in vitro* obtained cultures (Gantait and Kundu, 2007). These can be sown directly in soil or in different substrates such as sand, perlite or vermicompost. Direct sowing was achieved successfully in numerous species, such as *Medicago sativa* (Fuji et al., 1992), *Erythrina variegata* (Javed et al., 2017), *Dalbergia sissoo* (Chand & Singh, 2004), *Phyllanthus amarus* (Singh et al., 2006), *Musa* spp. (Ganapathi et al., 1992), *Morus indica* (Bapat & Rao, 1990), *Sphagneticola calendulacea* (Kundu et al., 2018).

One of the main limitations of using synthetic seed as a practical technology is the difficulty of sowing them directly in soil or in non-sterile substrates such as vermiculite, perlite, compost, etc (Rihan et al., 2012). The use of somatic embryos for large scale production is limited because of their asynchronous development, precocious germination and structural anomalies. However, this inconvenient can be overcome by using other type of explants for encapsulation, such as shoot tips, nodal segments and axillary buds.

In this context, the paper presents a study on the influence of sucrose from the encapsulation matrix, the effect of the salt concentration from the storage medium and the influence of sodi-

um alginate concentration on the storage capacity and viability of *Kalanchoe blossfeldiana* synthetic seeds.

MATERIALS AND METHODS

The material used for encapsulation was represented by nodal segments and shoot tips of *in vitro* grown *Kalanchoe blossfeldiana* plants, explants that were exercised after several proliferation subcultures on MS medium containing cytockinins (6-benzylaminopurine), auxins (indole-3-butyric acid) and gibberelines (gibberellic acid). Encapsulation was done in aseptical conditions, under the laminar hood.

For encapsulation, two variants of sodium alginate solution were used, both prepared in MS basal salt solution: V1 (with 2 % sucrose, v/w) and V2 (with 4 % sucrose, v/w), both prepared in MS basal salt solutions, in order to observe the influence sucrose has on the storage capacity of the explants. For the $CaCl_2$ solution, only one variant was used, in concentration of 100 mM. The pH of the sodium alginate solutions was modified to 5.8 and then the alginate and $CaCl_2$ solutions were sterilised in the autoclave at 121°C and 1.1 Bar atmospheric pressure for 20 minutes.

Explants with a small quantity of sodium alginate were dipped in the $CaCl_2$ solution using a glass pipette. Two immersion times were used for each variant: 10-15 minutes and 30-35 minutes. During the ion exchange time, the encpasulated explants were constantly stirred on the magnetic stirrer, at aprox. 10 rpm. After the ion exchange time ended, the encapsulated explants were rinsed three times with bidistilled sterile water, to remove any remaining traces of $CaCl_2$. The capsules formed after 30-35 minutes of immersion were isodiametrical and compact (Figure 1). Decreasing the immersion time to 10-15 minutes resulted in the formation of capsules that were fragile and more difficult to handle.

Synthetic seeds were stored in 3 variants of liquid MS storage medium. The liquid storage medium consisted of MS salts in different concentrations, with 3 % sucrose (v/w), în 3 variants: X (normal salts concentration), X/2 (halved salts concentration) and X/4 (quartered salts concentration). After preparation, the storage medium was sterilized in the autoclave, using the same protocol used for the alginate and $CaCl_2$ solutions.

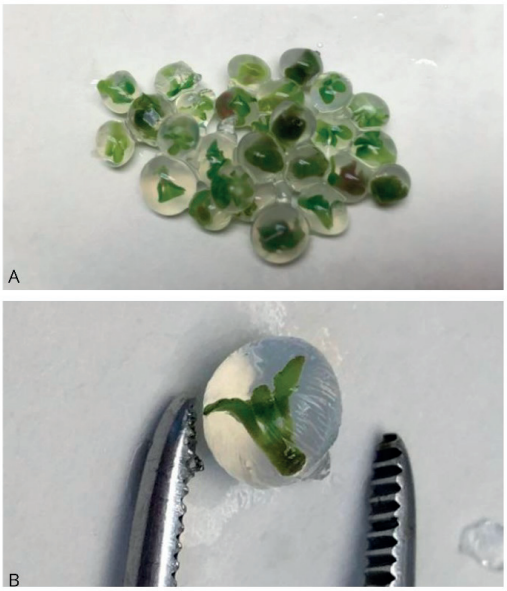

Figure 1. A, B: *Kalanchoe blossfeldiana* synthetic seeds after encapsulation (source: personal archive)

The encapsulated explants were kept în the liquid medium at 6°C and under, dark conditions. After the storage period (7, 30 and 60 days), the synthetic seeds were sown in *in vitro* conditions on basal hormone-free basal MS medium containing macronutrients, micronutrients, vitamins (Murashige & Skoog, 1962) and 3% sucrose (w/v) and solidified with 0.7% agar (w/v). Media pH was adjusted to 5.8 after the addition of sucrose and agar and was autoclaved at 1.1 Bar and 120°C for 20 minutes.

After inoculation on MS medium, synthetic seeds were transferred in the growing room at 22-25°C, with a 16 hours light/8 hours dark photoperiod and a 9.280 lx light intensity.

Observations reffering to rate of development, rooting percentage and average growth were taken at 7, 14 and 60 days of culture on hormone-free MS medium. Statistical analasys was applied in order to evidentiate any statistical differences in growth regarding the two factors analysed: the concentration of sucrose from the encapsulation, matrix and the concentration of MS salts from the storage medium. Bifactorial ANOVA test was applied to compare the means across the two independent variables:

the concentration of sucrose in the encapsulation matrix and the concentration of salts to evidentiate differences on average leaf number, followed by Tuckey's HSD test to see if the means significantly differ from each other. In case of growth, Kruskall Wallis (factor A - sucrose) and Mann-Whitney (factor B - MS salt concentration) tests were applied instead of two-factor ANOVA. Tukey HSD Post Hoc test was used to conduc a separate comparison between the variants.

Acclimatization started after 70 days of culture, using rooted shoots obtained from the synthetic seeds stored for 30 days in the frigde, at 6°C.
Rooted shoots (Figure 2) were removed from the *in vitro* environment and the agar was removed from the roots. These were placed in small pots, in sterilised substrate composed of peat and sand. The pots were sheltered with glass covers in order to keep the humidity level high.

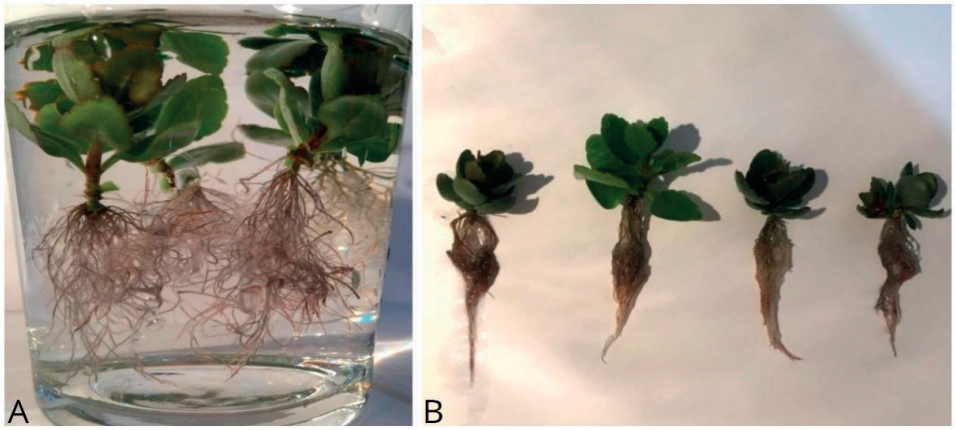

Figure 2. Rooted shoots of *Kalanchoe blossfeldiana* obtained from synthetic seeds, used for the acclimatization phase (source: personal archive)

RESULTS AND DISCUSSIONS

In vitro development of synthetic seeds

After seven days of storage: Observations on the development percetage were made 14 days after the innoculation of the synthetic seeds stored for seven days.
On average, synthetic seeds that were kept for 30 minutes in the $CaCl_2$ solution had a higher development rate (96.6 %) and viability, compared to the ones that were immersed for only 10 minutes (92.5 %).
All variants recorded 100 % development rate, except for variants V1 X/4 (10 minutes), V2 X/2 (30 minutes) and V1 X/4 (10 minutes), where the development varied between 75-80 % (Figure 3).

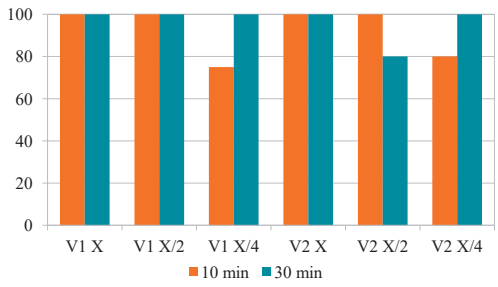

Figure 3. Development percentages of synthetic seeds stored for 7 days at 6°C.

After 30 days of storage: Development percentage after 14 days from sowing dropped from 96.6 % to 48.3 % when modifing the storage time from 7 to 30 days (Figure 4).

The highest development rates were recorded in the variants that were stored the medium with the quartered salt concentration (V1 X/4 and V2 X/4).

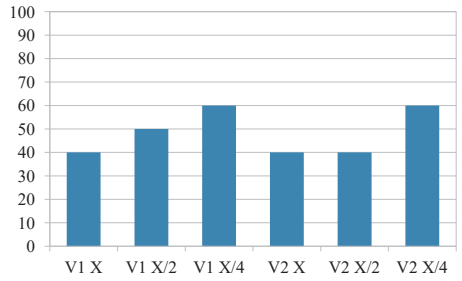

Figure 4. Development percentages of synthetic seeds stored for 30 days at 6°C

Rooting of synthetic seeds

Rooting took place simultaneously with the shoot growing, in the absence of hormonal treatments. Observations were made after 14 days of culture on hormone - free MS medium. The percentages of rooted explants were between 83 and 100% (Figure 5).

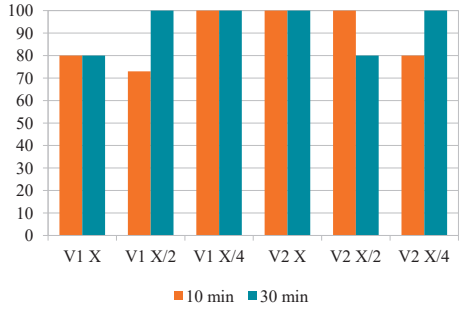

Figure 5. Percentages of rooted explants, after 14 days of culture on MS medium

Growth of synthetic seeds

Observations on growth were taken after 60 days of culture. This were recorded for the synthetic seeds immersed for 30 minutes in 100 mM $CaCl_2$ solution and stored for 30 days at 6°C. Mann-Whitney test revealed that modifing the sucrose concentration from the encapsulation matrix from 2% to 4% did not have a statistically significant influence on ulterior growth.

On the other hand, Kruskal-Wallis (Figure 6) test revealed significant differences between the stem growth regarding the salt concentration from the storage medium. The most favorable growth were recorded on the variants with the lowest salt concentrations: 1/4: 15.8 mm for V1 X/4 and 13.8 for V2 X/4, followed by the variants with the halved concentrations: 13 mm for V1 X/2 and 7.8 for V2 X/2. The variants with the normal salts concentration recorded the lowest growth: 5.4 mm for V1 X and 5 mm for V2 X (Figure 7).

	MS X	MS X/2	MS X/4	
median	5	10.5	15	
rank sum	66.5	163	235.5	
count	10	10	10	30
r^2/n	442.225	2656.9	5546.025	8645.15
H-stat				18.55032
H-ties				18.76321
df				2
p-value				8.43E-05
alpha				0.05
sig				yes

Figure 6. Kruskal-Wallis test results. Statistically significant differences between variants

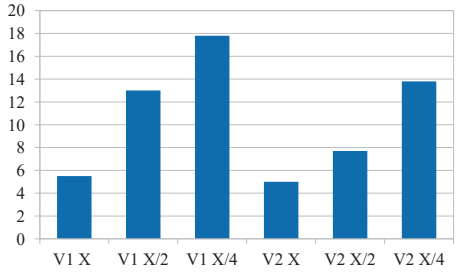

Figure 7. Average growth of synthetic seeds (mm), after 60 days of culture on MS medium

Average leaf number

Observations on average leaf number were recorded after 60 days of *in vitro* culture of the seeds immersed for 30 minutes and stored for 30 days at 6°C. Statistically, no differences between the average leaf number were recorded, for synthetic seeds encapsulated în V1 and in V2.

Statistically, significant differences were recorded between the storage mediums. Bifactorial ANOVA gave a significant result (p value =0.000198, <0.05), for factor A (concentration of MS salts in the storage medium), as a result the analysed means are not equal (Figure 8).

But in case of factor B (concentration of sucrose in the encapsulation matrix), p value (p=0.084445, >0.05) shows that there were no differences between the means (Figure 8).

ANOVA				Alpha	0.05	
	SS	*df*	*MS*	*F*	*p-value*	*sig*
Rows	83.46667	2	41.73333	12.52	0.000189	yes
Columns	10.8	1	10.8	3.24	0.084445	no
Inter	5.6	2	2.8	0.84	0.444012	no
Within	80	24	3.333333			
Total	179.8667	29	6.202299			

Figure 8. ANOVA test results

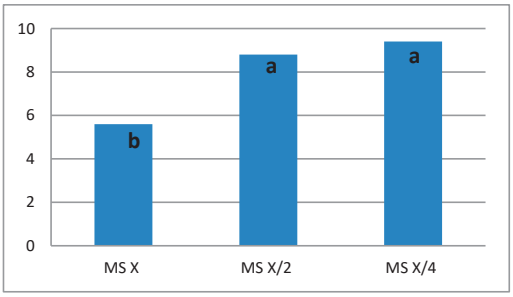

Figure 9. Tukey HSD results. Significant differences between MS X and MS X/2 & MS X/4 variants

The highest values were recorded in the variants with the quartered concentration,
followed by the ones with the halved concentration, with no significant differences between them, according to the results of Tukey HSD test (Figure 9).

Acclimatization of explants.

After two weeks of transferring the rooted shoots from *in vitro* to *in vivo* environment, the percentage of survival was 100 % for all encapsulation tested variants (Figure 10).

Figure 10. *In vitro*-grown acclimatized *Kalanchoe blossfeldiana* plants

CONCLUSIONS

Synthetic seeds stored for 1 week in MS salts liquid medium had development percentages between 75 and 100 %. On average, synthetic seeds that were kept for 30 minutes in the $CaCl_2$ solution had a higher development rate (96.6 %) and viability, compared to the ones that were immersed for only 10 minutes (92.5%).

Viability and development percentage decreesed from 96.6% to 48.3%, after increasing the storage time from 7 days to 30 days. Higher development percentages were recorded for the seeds that were stored in the medium with the quartered salt concentration (V1 X/4 and V2 X/4). The sucrose concentration did not influence the viability and growth of the synthetic seeds.

The concentration of MS salt in the storage medium did have a significant influence on the stem growth and leaf growth of synthetic seeds, as revealed by ANOVA and Kruskal-Wallis tests. Tukey's HSD test indicated that synthetic seeds stored in the medium with the lowest salt concentrations (X/2 & X/4) showed the highest average leaf growth.

No hormonal treatment is required in order to achieve rooting for synthetic seeds of *Kalanchoe blossfeldiana.* The development of the root system took place simultaneously with the shoot development. The percentages of rooted explants were between 73 and 100 % after 14 days of culture, but all shoots developed roots eventually, after 30 days of culture.

Acclimatization of the plants obtained from synthetic seeds was achieved successfully for all encapsulation tested variants.

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