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ENHANCING STORAGE CAPACITY OF POTATO SYNTHETIC SEEDS THROUGH THE USE OF SALICYLIC ACID

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Abstract

Solanum tuberosum is one of the most economically important species for food consumption. Because the species is susceptible to various systemic pathogens, in vitro techniques are preferred for storing the germplasm. Synthetic seed technology can be a useful tool in plant conservation, as it combines the advantages of vegetative and generative propagation. Using this technology, in combination with salicylic acid, a plant growth regulator known to mediate the plant response to cold temperatures, this study aims to enhance the tolerance of Solanum tuberosum explants to cold temperatures during in vitro storage. Nodal segments and shoot tips obtained from in vitro cultures of Solanum tuberosum 'Salad Blue' were encapsulated in sodium alginate solutions containing different concentrations of salicylic acid (0; 25 µM, 50 µM, and 75 µM) and stored at 4°C and under dark conditions for 60 days. Synthetic seeds were inoculated on a regeneration medium with 0.3 mg/L IAA and different concentrations of BAP (2 mg/L, 3 mg/L, and 4 mg/L). Even though the growth regulators in the culture medium did not influence the regeneration capacity of the explants, supplementing the alginate matrix with 25 µM salicylic acid increased the storage capacity of the encapsulated explants.

Key words*: artificial seeds, in vitro conservation, slow growth conservation, Solanum tuberosum.*

INTRODUCTION

Synthetic seeds are a type of artificial seeds that are created to emulate the structure of a natural seed. They are a type of slow-growth technique used for short and medium-term *in vitro* conservation of plant germplasm. The term was first introduced in 1977, by Toshio Murashige. Initially, it referred to only encapsulated somatic embryos (Murashige, 1977), but later, Bapat et al. (1987), has expanded the concept to non-embryogenic tissues. Non-embryogenic tissues that can be encapsulated include axillary or terminal buds, nodal segments, cell aggregates, or any other type of tissue that can develop into plants after short and medium periods of storage. Non-embryogenic tissues are preferred because somatic embryos have an asynchronous development and many species are recalcitrant to the process of obtaining them. However, they possess the ability to simultaneously develop shoots and roots, compared to other types of tissues (Ara et al., 2000; Micheli & Standardi, 2016; Magray et al., 2017). The advantages of cold storage in micropropagation are that it can diminish the cost of maintaining germplasm *in vitro*, as it minimizes manual labor and the number of subcultures (West et al., 2006) as well as costs associated with medium components, electricity, and space.

The applications of this technology are various, and include the multiplication of endangered species, elite genotypes, species where seed production is difficult or where the seeds are not true to type, or even commercially important species (Ray & Bhattacharya, 2008; Ghanbarali et al., 2016).

This technology has been successfully applied in *Solanum tuberosum*, by several authors, as it is an alternative that is very convenient to the conventional propagation of this species (Ghanbarali et al., 2016). Conventional conservation of potatoes is represented by storing tubers, which means they have to be grown annually, which is time-consuming (Roque-Borda et al., 2021). Germplasm conservation using true seeds is not an option, since this species is highly heterozygous and it produces seeds that are not true to type. However, since *Solanum tuberosum,* is a species

that originated in South America, in the subtropical biome, its capacity to withstand the low temperatures that are used within this technology, is limited. There are, nevertheless means of increasing the resistance to chilling temperatures in several species.

Cold storage using synthetic seed technology allows to storage of potato shoot tips for 180 days at temperatures of 4°C and 10°C, however, storing them at 4°C can successfully increase the storage time to 270 days, as the shoot tips will progressively turn brown faster at temperatures of 10°C, compared to 4°C (Nyende et al., 2003). Preculture of *Solanum tuberosum* 'Sante' and 'Agria' explants in MS medium supplemented with 10^{-6} M concentration of 24-epibrassinolide before the encapsulation of explants enhances the growth of axillary buds (Ghanbarali et al., 2016). 24-epibrassinolide (EBr) is a type of brassinosteroid, a growth regulator that was observed to influence a range of growth and development processes (Ghanbarali et al., 2016; Planas-Riverola et al., 2019) and to increase the tolerance to different types of abiotic stress, such as salt stress (Alam et al., 2019; Sousa et al., 2022), pesticide stress (Sharma et al., 2016), high and cold temperature stress (Xi et al., 2013; Chen et al., 2019; Fang et al., 2019). Direct sowing into *ex vitro* conditions, in soil, is possible using encapsulated nodal segments, with a survival rate of 57%, if treated with rooting powder before planting (Sarkar & Naik, 1998).

Another growth regulator with an important role in mediating the resistance to low temperatures is salicylic acid. Salicylic acid (S.A.) is renowned not just as a signal molecule mediating plant immunity, but also for its role as a plant growth regulator (Hayat et al., 2007; Rivas-San Vicente & Plasencia, 2011; Li et al., 2022). It has been demonstrated to have a role in mediating the plant response under different types of abiotic stress, for instance, salt stress (Idrees et al., 2012; Jayakannan et al., 2015) cadmium stress (Krantev et al., 2008; Kovács et al., 2014), drought stress (Bandurska & Stroi ski, 2005; Hayat et al., 2008; Chen et al., 2014), cold temperatures stress (Chen et al., 2020; Guo et al., 2023) and biotic stress (Emilda et al., 2020; Li et al., 2022).

There is numerous research that focuses on the influence of salicylic acid on cold temperature tolerance in several economically important species, for example, *Zea mays* (Li et al., 2017; Zhang et al., 2021), *Citrullus lanatus* (Jing-Hua et al., 2008), *Triticum aestivum* (Ignatenko et al., 2019; Wang et al., 2021), *Solanum melongena* (Chen et al., 2011) and *Spinacia oleracea* (Shin et al., 2018). Temperatures of 8°C are low enough to cause an increase in the endogenous levels of salicylic acid in *Cucumis sativus* seedlings (Dong et al., 2014). In wheat, the exogenous application of 100 µM salicylic acid enhances the activity of antioxidant enzymes and the accumulation of proline, increasing tolerance to cold temperatures (Ignatenko et al., 2019).

The exogenous treatment of leaves and roots with a 0.5 µM salicylic acid solution for one day can increase the chilling tolerance in sensitive banana seedlings (Kang et al., 2003).

In fruits, it has been regarded to enhance the tolerance to chilling injury of 'Hayward' kiwifruits by controlling the metabolism of hormones and proline and by maintaining the structure of the cell (Niu et al., 2024). In cucumber, salicylic acid has a critical role in the response of seedlings to chilling tolerance. (Dong et al., 2014) noted that the treatment of cucumber seedlings with inhibitors of salicylic acid biosynthesis will reduce the accumulation of endogenous S.A. and the plants have less tolerance to chilling injury.

Therefore, taking into account the multiple aspects previously reported, the purpose of this study is to observe the response of the explants of *Solanum tuberosum* 'Salad Blue' to the encapsulation technique and to assess the influence of salicylic acid on the cold storage capacity of the encapsulated explants.

MATERIALS AND METHODS

Preparation of encapsulation solutions and culture medium

For the encapsulation of *Solanum tuberosum* 'Salad Blue' explants, 4 variants of sodium alginate solutions were used, and one variant of calcium chloride solution.

The encapsulation matrix consisted of 3% (w/v) sodium alginate and 3% (w/v) D(+) Sucrose, prepared with basal MS macro elements, microelements, and vitamins, as described by (Murashige & Skoog, 1962). Three concentrations were employed to observe salicylic acid's influence on synthetic seeds'

storage capacity: 25; 50 and 75 µM. The composition of each variant of sodium alginate solution is detailed in Table 1.

Table 1. The composition of the sodium alginate solutions used for the encapsulation of explants

No	Code	Composition
1.	V0	MS components, 3% sucrose, 3% sodium alginate and no salicylic acid
2.	V1	MS components, 3% sucrose, 3% sodium alginate, and 25 µM SA
3.	V2	MS components, 3% sucrose, 3% sodium alginate, and 50 µM SA
4.	V3	MS components, 3% sucrose, 3% sodium alginate, and 75 µM SA

Regarding the hardening solution, only one variant of 100 mM $CaCl_2$, prepared in distilled water, was used. The regeneration medium for *Solanum tuberosum* 'Salad Blue' synthetic seeds consisted of basal MS macro elements, microelements, and vitamins, as described by Murashige and Skoog (1962), with 3% (w/v) D(+) Sucrose, 7 g/L Agar, in three variants, with three different concentrations of BAP (2; 3 and 4 mg/L) and 0.3 mg/L IAA, detailed in Table 2.

Table 2. The composition of the culture mediums used for the regeneration of synthetic seeds

No.	Code	Composition
1.	X0	MS components, 3% sucrose, 7 g/L agar, and no growth regulators
2.	X1	MS components, 3% sucrose, 7 g/L agar + 2 mg/L BAP and 0.3 mg/L IAA
3.	X2	MS components, 3% sucrose, 7 g/L agar + 3 mg/L BAP and 0.3 mg/L IAA
4.	X3	MS components, 3% sucrose, 7 g/L agar + 4 mg/L BAP and 0.3 mg/L IAA

The storage medium for low-temperature conservation contained MS salts in quartered concentration and 2.5 % (w/v) D (+) sucrose. The liquid medium was distributed approximately 40 ml in small jars of 100 ml total capacity

The pH of all culture mediums, storage medium, and sodium alginate solution was adjusted to 5.75 and then sterilized in the autoclave at 121°C and 1.1 bar atmospheric pressure for 20 minutes.

Encapsulation of explants

The biological material used for the experiment 'Salad Blue' shoots (Figure 1) from *in vitro* cultures, maintained in the Plant Micropropagation Laboratory of the Research Center for Studies of Food Quality and Agricultural Products from the University of Agronomic Sciences and Veterinary Medicine of Bucharest. Before encapsulation, nodal segments were grown for 2 months on Murashige and Skoog medium (Murashige & Skoog, 1962), without any growth regulators.

Figure 1. *In vitro* obtained shoots of *Solanum tuberosum* 'Salad Blue' used for encapsulation

The *in vitro* grown shoots were cut into approximately 2-3 mm long nodal segments, with at least one axillary bud present (Figure 2), and placed in the sodium alginate solution.

Figure 2. Nodal segments of *Solanum tuberosum* 'Salad Blue' prepared for encapsulation

The explants, together with a small quantity of the sodium alginate solution, were dipped into the calcium chloride solution using a glass pipette. The $CaCl_2$ solution containing the explants was constantly stirred (on a magnetic stirred, at approximately 10 rpm) during the ion exchange process, to allow the formation of isodiametric capsules. After 13 minutes, the encapsulated explants were rinsed three times with sterile distilled water to remove any remains of the $CaCl_2$ solution. After rinsing, the

capsules were dried for a few minutes on sterile filter paper (Figure 3) and then placed in small jars containing the conservation medium.

Figure 3. Encapsulated nodal segments of *Solanum tuberosum* 'Salad Blue'

The storage medium for low-temperature conservation contained MS salts in quartered concentration and 2.5% (w/v) D (+) sucrose. Synthetic seeds were stored in the conservation medium, for 60 days, at 4°C, under dark conditions. After 60 days, the synthetic seeds were inoculated on the regeneration mediums, as detailed in Table 3. The synthetic seeds were transferred into the growing room, at a temperature of 22-25°C, 5023 lx light intensity using white, red, and blue light-emitting diodes (LEDs) and with a 16 hours light and 8 hours darkness photoperiod.

Statistical analysis

For the statistical analysis, The Real Statistics Resource Pack (https://real-statistics.com/) for Excel 2019 was used. Because the sample size was not equal for all variants, the Kurskal-Wallis test was used for the analysis of variance instead of ANOVA.

Table 3. Regeneration medium for *Solanum tuberosum* 'Salad Blue' synthetic seeds with different concentrations of 6-benzylaminopurine (BAP) and Indole-3-acetic acid (IAA)

Medium variant	Growth regulators concentrations	Control - 0 µM S.A. (V0)	Variant 1 - 25 µM S.A (V1)	Variant 2 - 50 µM S.A (V2)	Variant 3 - 75 µM S.A (V3)
X0	BAP (mg/L)	0	2	3	4
	IAA (mg/L)	0	0.3	0.3	0.3
X1	BAP (mg/L)	0	2	3	4
	IAA (mg/L)	0	0.3	0.3	0.3
X2	BAP (mg/L)	0	2	3	4
	IAA (mg/L)	0	0.3	0.3	0.3
X3	BAP (mg/L)	0	2	3	4
	IAA (mg/L)	0	0.3	0.3	0.3

RESULTS AND DISCUSSIONS

Considering the total number of seeds encapsulated in each variant of salicylic acid (V0 - 0 µM, V1 - 25 µM, V2 - 50 µM and V3 - 75 µM), the highest percentage of regeneration was observed in variant V1 with 25 µM salicylic acid (78.72%), followed by the variant V3 with 75 µM salicylic acid (58.33%), V0 - 0 µM salicylic acid (51.06%) and V2 with 50 µM salicylic acid (43.75%), as it can be observed in Figure 4.

Generally, the highest regeneration percentages of 91.67% were achieved in variant V1X1 (25 µM on medium with 2 mg/L 1 BAP and 0.3 mg/L IAA), variant V1X3 (25 µM on medium with 4 mg/L and 0.3 mg/L IAA), and variant V0X0 (0 µM S.A. and no hormones in the regeneration medium), as depicted in Figure 5.

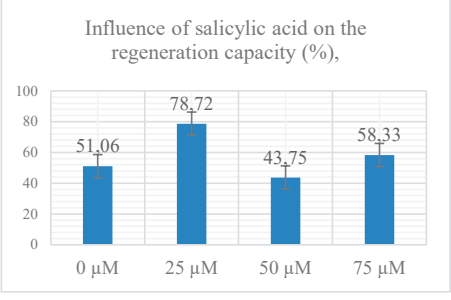

Figure 4. Influence of the concentration of salicylic acid on the regeneration of synthetic seeds observed two weeks after inoculation on medium

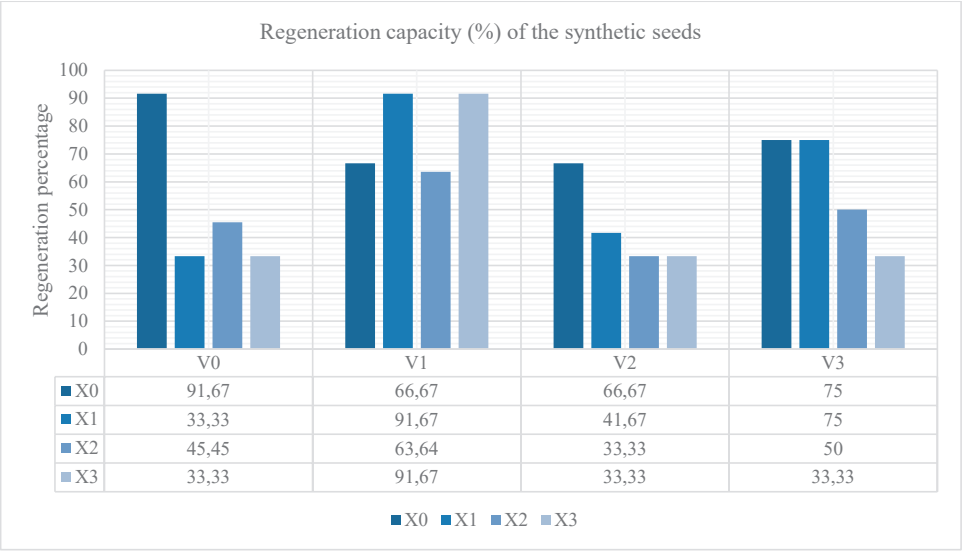

Figure 5. The regeneration capacity (%) of the synthetic seeds, observed two weeks after inoculation on the regeneration medium

Regarding the concentrations of growth regulators present in the growing medium, the highest percentage of regeneration was recorded on X0, the control medium, with no growth regulators (81.25%). Adding IAA and different concentrations of BAP decreased the speed of regeneration from 60.42% in the X1 variant (2 mg/L BAP + 0.3 mg/L IAA) to 47.92% in variant X3 (4 mg/L BAP and 0.3 mg/L IAA) and to 47.83% in variant X2 (mg/L BAP and 0.3 mg/L IAA), accordingly to Figure 6.

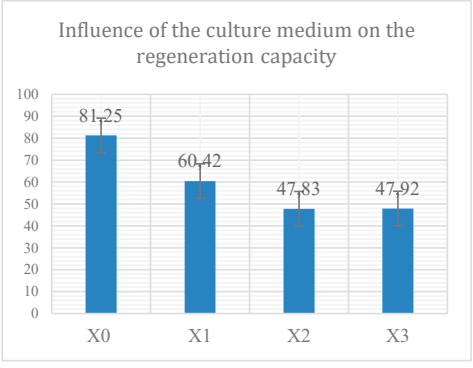

Figure 6. Influence of the concentration of growth regulators on the regeneration of synthetic seeds, observed two weeks after inoculation on medium

It must be noted, that all explants inoculated on the mediums with growth regulators were able to regenerate shoots, but at a very low speed, because during the first month of culture, the tissue at the base of the explants started dedifferentiate and produce callus cells.

Growth of synthetic seeds

Average shoot length (mm)

The highest values regarding the average shoot length seeds were recorded in the variants of synthetic seeds that were sown on hormone-free medium: 56.18 mm (V3X0 - 75 µM S.A.), 54.62 mm (V1X0 - 25 µM S.A.), 54.44 mm (V2X0 - 50 µM) and 49.32 mm (V0X0 - 0 µM). Overall, the lowest average growth values were recorded in the variants that were sown on the mediums with the highest concentrations of BAP (X): 4 mg/L BAP and 0.3 mg/L IAA (Figure 7). Kruskal-Wallis revealed statistical differences between the analyzed variants.

Regarding the overall hormone concentrations of the regeneration medium, the Kruskal-Wallis test revealed significant differences between the four variants, with growth factor declining with increasing hormone concentrations, with the highest value of 54.31 mm on the hormone-free medium (X0) and the lowest value of 22.45 mm on the medium with 4 mg/L BAP and 0.3 mg/L IAA (Figure 8 A). No statistically significant differences were recorded in the variants sown on medium with 2 mg/L BAP and 0.3 mg /L IAA (X1) and 3 mg/L BAP and 0.3 mg/L IAA (X2).

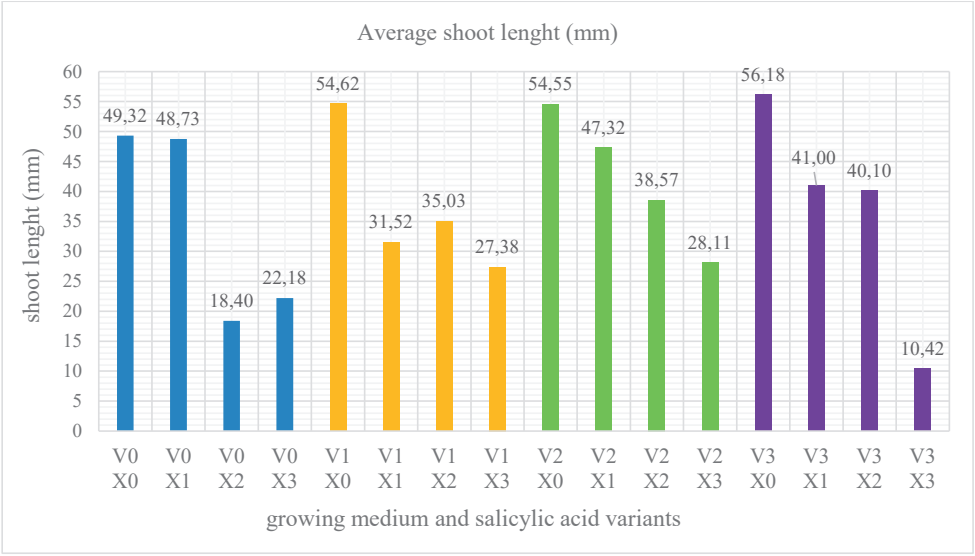

Figure 7. Average length (mm) of the shoots regenerated from the encapsulated explants

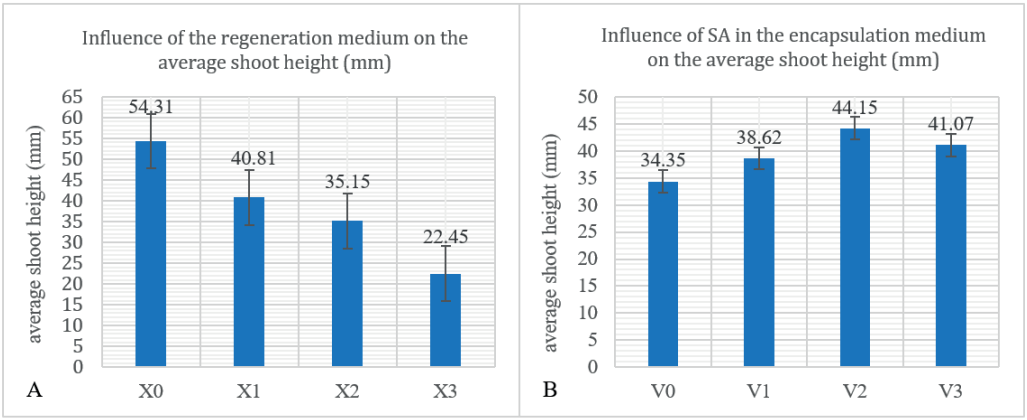

Figure 8. Influence of the growth regulators in the regeneration medium on the average shoot height (A). Influence of the encapsulation matrix on the average shoot height (B)

If analyzed by the concentration of salicylic acid in the medium, the Kruskal-Wallis test reveals a p-value > 0.05, pointing out no statistically significant differences regarding the average shoot length.

The highest average value of 44.15 mm was obtained for the explants encapsulated in the variant with 50 µM SA, followed by 41.07 mm on the variant with 75 µM, 38.62 mm on the variant with 25 µM and the lowest value, 34.35 mm on the control variant.

CONCLUSIONS

Even though it originates from a subtropical biome, *Solanum tuberosum* is a species that has a good potential to be conserved using *in vitro* cold slow-growth techniques and synthetic seed technology. Because conventional conservation of this species through tubers is time-consuming and not economical, and because conservation through seeds is not possible, the development of other conservation protocols is important.

Encapsulation of explants of *Solanum tuberosum* using synthetic seed technology can also ensure that the material that is regenerated from them is free of pests and diseases, and, compared to other *in vitro* conservation methods, such as cryopreservation, it is less expensive and requires less specialized equipment.

Supplementing the alginate matrix with 25 µM salicylic acid increases the regeneration capacity to 78.72% in synthetic seeds of 'Salad Blue'. Sharifeh et al. (2011), obtained similar results, where supplementing the alginate matrix with 25 µM or 50 µM salicylic acid increases the viability of *Helianthus annus* synthetic seeds, after 90 days of storage.

Regarding the regeneration medium, growth regulators are not mandatory for the regeneration of 'Salad Blue' nodal segments, as our study has shown that it decreases the speed of regeneration and the height of the shoots, as the concentrations of BAP and IAA used in this experiment stimulated more the dedifferentiation of cells and callus growth, than the regenerations of shoots.

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