Contents lists available at *Dergipark*

Journal of Scientific Reports-A

journal homepage: *https://dergipark.org.tr/tr/pub/jsr-a*

E-ISSN: 2687-6167 ***Number 57, June 2024***

RESEARCH ARTICLE

Receive Date: 09.02.2024 *Accepted Date: 25.03.2024*

A research on the production, storage and germination of synthetic seeds in tea plant (*Camellia sinensis* [L.] O. Kuntze)

Begüm Güler[a,*], Aynur Gürel[b]

[a]*Kırşehir Ahi Evran University Faculty of Engineering and Architecture, Genetic and Bioengineering Department, Kırşehir, Turkiye,*
ORCID: 0000-0002-9970-2111
[b]*Ege University, Faculty of Engineering, Bioengineering Department, İzmir, Turkiye,*
ORCID: 0000-0002-7002-9752

Abstract

Tea plant, is one of the most popular beverages consumed worldwide because of its rich and pleasant flavors and numerous health benefits. In this study, we performed production, storage and germination of synthetic seeds in the tea plant by encapsulation of somatic embryos. In our research, after the encapsulation of the mass-produced embryogenic calli with different doses of sodium alginate (NaAlg) and $CaCl_2$, they were stored at different temperatures and at different times, and then transferred to different nutrient media after the expiry of the different storage period to determine the most suitable nutrient composition for germination. The resulting embryogenic calli were stored after encapsulation and then transferred to germination media. Although there was very little germination in long-term storage, the data obtained were found to be statistically insignificant. The germination rate of the beads, which were transferred directly to the germination medium without storage and encapsulated using 3% Na-Alg and 50 mM $CaCl_2.2H_2O$, was determined as 44.44% in the MS medium containing 3 mg/L BAP and 1 mg/L IBA. In addition, it was observed in the study that increasing storage time increased the darkening of the beads, while increasing NaAlg and $CaCl_2$ doses caused obtaining harder and more nontransparent beads. Hyperhydricity problem was not encountered in any trial in the study. This study, carried out with our local tea variety.

Keywords: Tea plant; synthetic seed; encapsulation; somatic embryo; storage period

* Corresponding author. Tel.: +90-386-280-2256;
E-mail address: begum.guler@ahievran.edu.tr

1. Introduction

Tea plant (*Camellia sinensis* [L.] O. Kuntze), which belongs to the Theaceae family, is one of the most important economic plants in the world due to its numerous medicinal benefits and being the most consumed product as a beverage [1]. The tea plant, which is a perennial and evergreen plant, also has many medicinal properties. Black tea, obtained by brewing processed leaves, is the most consumed beverage all over the world. Medicinal use of tea dates back 4700 years. Today, it is included in the daily diet of people in many regions, especially in Asia. Apart from China, India, Sri Lanka, Kenya, Japan, Indonesia, Malawi and Turkiye are the largest tea producers in the World [2].

Tea propagation is carried out in two different ways: generative (seed) and vegetative (cutting, grafting and tissue culture) [3]. Most of its production is carried out with seeds. However, seed production causes problems such as genetic variations, yield differences and changes in quality characteristics [4]. For this reason, seed production is not one of the preferred production techniques. Vegetative techniques also cause various problems. Due to reasons such as dormancy and drought, there are no suitable seedlings in some tea gardens. Low rooting percentage and reproduction rates similarly constitute disadvantages of vegetative production techniques. When these disadvantages are examined, tissue culture applications stand out as the most effective production technique among all production methods [3].

Among tissue culture techniques, synthetic seed technology (also called “synseed” or “artificial seed”) is a valuable alternative technique for micropropagation and conservation of important agroeconomic plants [5]. Synthetic seed is defined as the synthetic encapsulation of somatic embryo, shoot tip, cell aggregate or other tissues that has the potential to turn into a full plant under *in vitro* and *ex vitro* conditions and can continue to show this feature after long-term storage [6]. “Synthetic seed technology” has many advantages, such as providing a protective coating that encapsulates the reproductive structures with the necessary nutrients, maintaining their strong and high adaptability during storage, and providing ease of packaging, storage and transportation [7]. With the use of synthetic seeds, the regenerable meristematic parts within the synthetic seed can be stored for different time periods and also ensure mass multiplication and preservation of germplasm [8].

In general, plant seeds have embryos containing one or two cotyledons connected to the endosperm, which serves as a nutrient reservoir for the development of the embryos. This structure is covered with a hard structure called testa, which protects the inner sensitive structure from injuries and ensures that the embryo remains alive until the germination period [9]. Although synthetic seeds have similar properties to natural seeds, there are also some distinct differences between them. T. Murashige put forward the first idea about synthetic seeds in 1977 [10]. He first expressed his idea about synthetic seeds at the "Symposium on the Tissue Culture for Horticultural" held in Genth, Belgium, on 6-9 September 1977. In this speech, while talking about the features that synthetic seed technology should have, the researcher stated that the production method should be extremely fast, allow a large number of production in a day, and should be competitive with the seed production technique [6]. The first synthetic seed production was carried out by Kitto and Janick in carrot somatic embryos in 1982. In this study, polyoxyethylene was used as the coating material [9].

Although somatic embryos were generally used in the first studies on synthetic seeds, many plant parts are used for encapsulation purposes today. Somatic embryos are followed by shoot tip and axillary shoots, respectively. Protocorm-like structures, nodal segments, and callus are also explant sources used in the production of synthetic seeds. Plant lines obtained through somatic embryos can maintain their regeneration capacity for a very long time, and also prevents dedifferentiation at the callus stage, thus ensuring the preservation of the genetic structure and the formation of a uniform genetic structure. [6, 11]. The synthetic seed containing the somatic embryo can be encapsulated directly into the synthetic shell, or it can be coated after being partially dehydrated [5]. Somatic embryos continue to form in cell masses and thus it is possible to produce several thousand embryos per gram in a culture. This makes somatic embryos a good source. Since the plant parts used in synthetic seed production are generally produced by *in vitro* clonal propagation techniques, they do not include meiotic recombination (during crossing-over) and gametic fusion (during cross-pollination), which are the two basic steps of sexual reproduction.

For this reason, new plants produced through synthetic seed technology have characteristics true to their name. Synthetic seeds are covered with a coating material that will provide the protection needed during storage, shipping and transportation. In some cases, this coating material may also contain plant nutrients and plant growth regulators [12].

Dried artificial seeds are naked. The drying process can be carried out quickly or by leaving it to dry overnight in lidless petri dishes or slower under more controlled conditions by reducing the humidity. In addition to the use of high osmotic potential, sublethal stress conditions such as nutrient deprivation or low temperature are used to induce tolerance to desiccation. High gel strength or osmotic agents such as mannitol or sucrose are used to increase the osmotic potential [6, 9]. Hydrogel capsules are a method used to encapsulate plant parts that are recalcitrant and sensitive to drying [6]. The method may also vary depending on the type of encapsulation preferred in synthetic seed production. While only a single coating layer is used in the single-layer encapsulation method, a second coating is applied on the coated material in the double-layer coating technique. The hollow beads technique is generally not preferred because it is laborious and costly, and the seeds cannot be protected well enough [13].

For the purpose of synthetic seed production, it has been determined that the most suitable material among many coating materials such as agar, alginate, carboxyl methyl cellulose, sodium pectate, gelrite, guar gum, etc. is alginate [6, 9]. Generally, 100 mM Ca^{+2} is used as a complementary solution with 2% NaAlg. This composition creates a protective layer as well as nutritional elements, and the seeds are easier to use and store. The basic principle in performing alginate encapsulation is based on the displacement of Na^{+} ions in NaAlg and Ca^{+2} ions in $CaCl_2.2H_2O$ solution when NaAlg solution containing plant parts is dropped into $CaCl_2.2H_2O$ solution. Endosperm-like structure formed as a result of coating can be used to carry microorganisms, nutrients, antibiotics, plant growth regulators, pesticides and fungicides. Endosperm may contain plant growth regulators and carbon sources not only for the physical protection of embryos but also for germination and plant growth [6]. In encapsulation, the coating material may contain nutrients, biological fertilizer, pesticides, nitrogen-fixing bacteria, antibiotics or other necessary components [14]. In a study conducted by Khor anh Loh, they determined that the transformation potential and viability of synthetic seeds increased when the coating material and activated carbon were used together [15]. They attributed this to the fact that activated carbon breaks the alginate and the embryo in the synthetic seed can breathe more easily in this way. The covering material should not harm the embryo, allow germination, but should be resistant to the difficulties encountered during production, storage, transportation and planting processes, as well as providing nutrients to the embryo for germination [9].

The reason why alginate is frequently preferred is its suitable viscosity, low toxicity, fast gelling and low cost. It also better protects the explant it covers against mechanical damage. The state of the encapsulated beads depends on the concentrations of NaAlg and calcium chloride, but also on the mixing time based on the plant species and explant source [11]. Redenbargh et al. stated that alginate hydrogels are the most suitable encapsulation material with their properties such as light viscosity, low toxicity and rapid gelation [16]. Nair and Reghunanth determined that 4% NaAlg and 100 mM $CaCl_2.2H_2O$ were coating materials of similar quality and suitability for *Clitoria terretea* and *Indigofera tinctoria*, respectively [17, 18]. At low concentrations of NaAlg and $CaCl_2$, irregularly shaped, brittle beads are formed, while at high concentrations, beads with a hard structure that do not allow germination are formed [19]. Mohanraj et al. determined that the use of high NaAlg reduced the germination frequency of beads [20]. In their study, Lulsdorf et al., found a high recycling rate after 4 weeks of storage at $4^{o}C$. Similarly, [22] stored synthetic seeds obtained from olive somatic embryos at 2 or $4^{o}C$ for 23 months and determined a germination rate of 61% [21].

Synthetic seed applications have many advantages. By directly encapsulating and using somatic embryos, the number of subculture processes required for the production of plants obtained from embryos through regeneration is reduced. Additionally, meiotically unstable elite genotypes can be protected with adjuvants such as plant growth regulators and pesticides. In addition to determining the role of seed coat formation and endosperm in embryo development and germination, synthetic seed technologies can be used to obtain information on plant development. While allowing large-scale production, synthetic seeds can also be used for germplasm preservation, medium-long

term or cold storage and disease eradication. Seeds can be produced in every season and every period, regardless of the season. Seed production can be achieved in a very short time and it is extremely easy to apply and has low cost. Seed production can be achieved with low cost and high efficiency [6, 12, 23].

In addition to their many advantages, synthetic seeds also have some disadvantages. The biggest problems experienced in synthetic seed technology are listed as the need to improve storage conditions due to dormancy problems, problems in ensuring synchronization in somatic embryo regenerations, improper maturation, low conversion rate to plants, problems in the acclimatization of weak plants due to lack of lignification and low cuticle formation [6]. Additionally, the rooting needs of non-embryogenic plant parts indicate the need for further research, especially in woody plants [11].

In a study conducted by Seran et al., with zygotic embryos of the tea plant, synthetic seeds coated with 2, 3, 4% (w/v) NaAlg and 100 and 50 mM $CaCl_2.2H_20$, 3 mg/L BAP and 0.5 mg /L was germinated on MS media with and without IBA [24]. As a result of the study, it was determined that synthetic seeds produced with 3% NaAlg + 100 mM $CaCl_2.2H_2O$ had the most suitable coating form, and for germination, MS medium containing growth regulators showed a higher germination rate [24].

In this study carried out by us, it was aimed to produce synthetic seeds and determine appropriate storage and germination conditions. For this purpose, encapsulation process was carried out with different ratios of NaAlg and $CaCl_2$, the produced beads were stored for different periods and conditions and germinated in different nutrient media. There are somatic embryogenesis studies in the tea plant. In addition, different explant sources were used in synthetic seed studies in tea. Our study is a pioneer in the production of synthetic seeds in tea plants by encapsulation of somatic embryos.

2. Methods

2.1. Material

In the studies, leaf explants of the *Camellia sinensis* [L.] O. Kuntze cv. Ali Rıza Erten were used to produce somatic embryos. Ali Rıza Erten variety is a local variety developed by Rize Tea Research Institute. Tea plantlets under *in vitro* conditions were purchased from Dikili Ciftlik company and propagated clonally.

2.2. Production of somatic embryos

40-day-old plantlets under *in vitro* conditions were subcultured in a laminar flow cabinet. To ensure the continuity of stock plants during this subculture process, shoot tip and node explants were cultured in MS [25] medium containing 0.6 mg/L BAP and 0.1 mg/L NAA, while leaf explants were injured and transferred to MS medium containing 4 mg/L BAP, 1.5 mg/L IBA and 30 g/L sucrose. After callus initiation was achieved in this environment, the callus clusters were transferred to MS medium containing 4 mg/L BAP, 1.5 mg/L IBA and 50 g/L sucrose for secondary embryogenesis. Embryos with secondary embryogenesis observed in nutrient medium and at the globular stage were used in the production of synthetic seeds.

2.3. Production of synthetic seeds

In synthetic seed experiments, a pasteur pipette with a cut off tip was used to form beads. To sterilize the Pasteur pipette, its entirety (including its inner part) was treated with plenty of 70% ethyl alcohol solution and then subjected to UV sterilization for 15 minutes. Then, a large amount of bleach (commercial bleach containing 5% NaOCl) was taken into a straw in the cabin and allowed to come into contact with all surfaces. To remove the bleach, rinse with sterile distilled water until the foam was completely gone. It was decided that this process was sufficient for surface sterilization of the Pasteur pipette.

As a result of the literature research [24, 26], it was decided to use 50 mM and 100 mM $CaCl_2$ and 3, 4 and 5% NaAlg for coating purposes. After the embryos were separated from each other in the cabin with the help of a scalpel, they were transferred to autoclaved NaAlg solution. After all embryos were transferred to the NaAlg solution, they were dropped into the $CaCl_2$ solution in a beaker on the magnetic stirrer with the help of a Pasteur pipette with the tip cut off and sterilized with bleach in the cabinet. Mixing with orbital shaker was continued for 30-40 minutes for the beads to harden. At the end of 40 minutes, the $CaCl_2$ solution was removed with the help of a sterile filter and the beads were rinsed 3 times with sterile distilled water to remove excess $CaCl_2$. Then, the beads were placed on a sterile napkin and waited for a short time (1-2 min) to remove excess water. After this process, if the beads were to be stored, they were placed in a sterile petri dish and stored in the place where they would be stored, or they were transferred directly to germination media.

2.4. Storage and germination of synthetic seeds

The obtained beads were stored in dark conditions at 4°C (refrigerator) and 25°C (cabinet) for 0, 15, 30 and 60 days to determine the appropriate storage condition. As a result of the storage period, the synthetic seeds were transferred to the designated germination media (Table 1). The culture vessels were placed on culture shelves at 24±2°C under 16-hour light/8-hour dark photoperiod conditions to ensure germination. Observations were made once a week and the cracking of the beads and the conditions of the embryos were noted. The experiments were set up according to the fully randomized trial design and were planned with 3 replications, with 3 beads in each replication. Minitab 17 Statistical Software (Minitab Inc, PA, USA) was used to evaluate the data obtained.

Table 1. Germination media compositions*.

Code	Germination media
ÇG1	0.5 mg/L GA3
ÇG2	1 mg/L GA3
ÇG3	1.5 mg/L GA3
ÇG4	3 mg/L BAP + 0.5 mg/L IBA
ÇG5	3 mg/L BAP + 1 mg/L IBA
ÇG6	3 mg/L BAP + 2 mg/L IBA
ÇG7	2 mg/L BAP + 1 mg/L IBA
ÇG8	1 mg/L BAP + 1 mg/L IBA
ÇG9	0.5 mg/L BAP + 1 mg/L IBA

***** In all experiments, MS (Murashige and Skoog, 1962) was used as the basic nutrient medium, 30 g/L sucrose as the carbon source, and 3 g/L Gelrite as the gelling agent. pH:5.8

2.5. Statistical evaluation of data

All *in vitro* treatments were applied in a randomized plot design with three replications. The data obtained from the applications were evaluated with the Minitab 17 (Minitab®, LLC, Pennsylvania, USA, 2015) program.

3. Results

The synthetic seeds obtained in the study were coated using different concentrations of NaAlg and $CaCl_2$. The obtained beads were stored at different temperatures for different periods of time and transferred to different nutrient media in order to crack the beads as a result of the storage period. By determining the cracked (germinated) beads of these beads, an attempt was made to determine the most suitable NaAlg and $CaCl_2$ concentration, the most suitable storage time and temperature, as well as the most suitable nutrient medium. Due to the intense amount of contamination seen in the embryogenic calli of the tea plant, the problem that these contaminations can remain hidden for a long time and emerge in unexpected situations has been encountered in many studies [27]. A similar

situation was encountered in this study conducted by us. While no contamination was observed in the previous stages of the study, contamination occurred during the storage of synthetic seeds. While a higher rate of contamination was observed especially in beads stored at 24°C, it was determined that both contamination and darkening were less in beads stored at 4°C. Increasing storage time also caused the darkening problem to be observed. In addition, since the size of the beads formed is related to the diameter of the pipette, it was observed that their sizes were distributed in the range of 3-5 mm on average. Preliminary studies showed that the mixing process should be fast enough to prevent the beads from piling up and sticking, but not so fast as to create shear stress on the bead surface and prevent its shape from becoming more elliptical. In addition, it was determined that the coldness of the $CaCl_2$ solution used had an effect on the beads having a more stable round shape/hard structure, and therefore keeping them at 4°C overnight after the autoclaving process was important for bead formation (Fig 1).

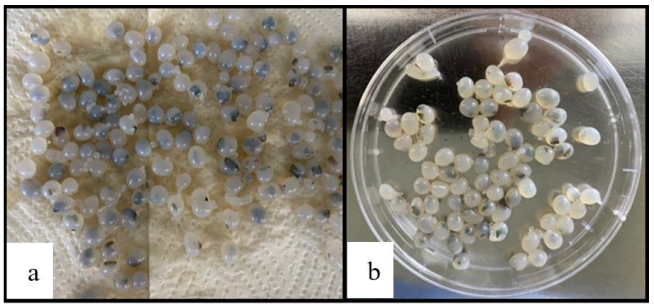

Fig 1. Synthetic seeds a) beads after rinsing, b) beads ready for storage.

In the study, data regarding the percentage of germinating beads were examined. When NaAlg values were examined, the highest germination rate was detected in 3% trials (2.46%). At $CaCl_2$ doses, the highest germination rate of 1.54% was obtained in the 50 mM experiment. When the storage periods were evaluated, the highest germination rate was observed in the trials with no storage (0 day), while the data obtained was not found to be statistically different, although there was germination (0.21%) in the 60-days trials (Table 2). As a result of the experiments carried out within the scope of the study, CaCl2 doses and various interactions were also examined. When the relationship between NaAlg and $CaCl_2$ was examined, 4.63% germination rate occurred in beads produced with 3% NaAlg and 50 mM $CaCl_2$. When all trials were evaluated, the highest germination rate of 6.17% was obtained in trials without storage. When 100 mM and 50 mM trials were compared, the highest germination rate (1.54%) was detected in 50 mM $CaCl_2$ trials. There was no statistically significant difference between the data obtained at different storage temperatures.

Table 2. Data on the binary interactions of different $CaCl_2$ doses on the percentage (%) of germinating (cracked) seeds in synthetic seed experiments*.

	C	50 mM	100mM	Average
A	3%	4.63 a	0.31 b	2.46 a
	4%	0.00 b	0.92 b	0.46 b
	5%	0.00 b	0.00 b	0.00 b
S (days)	0	6.17 a	1.23 b	3.70 a
	15	0.00 b	0.00 b	0.00 b
	30	0.00 b	0.00 b	0.00 b
	60	0.00 b	0.41 b	0.21 b
Average		1.54 a	0.41 b	

* Applications were performed in triplicate and 3 beads were used for each replication. The differences between the values indicated with different letters are significant at the p<0.0.1 level according to the Duncan multiple comparison test. A: NaAlg dose, C: $CaCl_2$ dose, S: Storage time

In this study, comparisons with different interactions were also examined to determine the effect of media

composition on germination. When the media composition and NaAlg interaction were examined, the best germination percentage (5.55%) was observed in ÇG5 medium in 3% NaAlg trials. When media composition and $CaCl_2$ interaction were evaluated, the best result was found in the ÇG5*50 mM trials (3.70%). When the relationship between media composition and storage time was examined, it was concluded that storage reduced germination. In trials without storage, 7.41% germination rate was achieved in ÇG5 medium. Although 0.92% germination rate occurred in 60-day trials on ÇG8 medium, the difference was not statistically significant (Table 3). When the media compositions were evaluated in general, it was determined that the most suitable germination medium was MS medium (ÇG5) containing 3 mg/L BAP and 1 mg/L IBA. As a result of the analysis of variance performed, NaAlg dose (A), $CaCl_2$ dose (C) and storage time (S) were found to be statistically significant. Additionally, A*C, A*S, A*medium composition (M), C*S, C*M, A*C*S, A*S*M, C*S*M and A*C*S*M interactions were also found to be statistically significant. When all conditions were examined in detail, synthetic seeds coated with 50 mM $CaCl_2$ and 3% NaAlg gave the best results with a germination rate of 44.44% in conditions without storage in ÇG5 medium (Table 4).

Table 3. Data on the binary interactions of percentage (%) germination (cracking) bead rates in synthetic seed experiments in different media*.

M	A			T		C		S (days)			Average
	%3	%4	%5	4°C	24°C	50 mM	100 mM	0	15	30	60	
ÇG1	1.39 bcd	0.00 d	0.00 d	0.46	0.46	0.92 bc	0.00 c	1.85 cd	0.00 c	0.00 c	0.00 c	0.46 ab
ÇG2	2.78 abcd	0.00 d	0.00 d	0.92	0.92	1.85 abc	0.00 c	3.70 bc	0.00 c	0.00 c	0.00 c	0.93 ab
ÇG3	0.69 cd	0.00 d	0.00 d	0.00	0.46	0.00 c	0.46 c	0.00 c	0.00 c	0.00 c	0.92 cd	0.23 ab
ÇG4	4.17 ab	0.00 d	0.00 d	1.39	1.39	2.77 ab	0.00 c	5.55 ab	0.00 c	0.00 c	0.00 c	1.39 ab
ÇG5	5.55 a	0.00 d	0.00 d	1.85	1.85	3.70 a	0.00 c	7.41 a	0.00 c	0.00 c	0.00 c	1.85 a
ÇG6	4.17 ab	0.00 d	0.00 d	1.39	1.39	2.77 ab	0.00 c	5.55 ab	0.00 c	0.00 c	0.00 c	1.39 ab
ÇG7	0.00 d	0.00 d	0.00 d	0.00	0.00	0.00 c	0.00 c	0.00 c	0.00 c	0.00 c	0.00 c	0.00 b
ÇG8	3.47 abc	0.00 d	0.00 d	1.39	0.92	1.85 abc	0.46 c	3.70 bc	0.00 c	0.00 c	0.92 cd	1.16 ab
ÇG9	0.00 d	4.17 ab	0.00 d	1.39	1.39	0.00 c	2.77 ab	5.55 ab	0.00 c	0.00 c	0.00 c	1.39 ab

*Applications were performed in triplicate and 3 beads were used for each replication. The differences between the values indicated with different letters are significant at the p<0.0.1 level according to the Duncan multiple comparison test. M: Medium code, A: NaAlg dose, C: $CaCl_2$ dose, S: Storage time, T: storage temperature

Table 4. Data on the percentage (%) of germinating (cracked) beads in synthetic seed trials*.

ACT			3%					4%					5%					
			50 mM		100 mM		Avg	50 mM		100 mM		Avg	50 mM		100 mM		Avg	AVG
		M	4°C	24°C	4°C	24°C		4°C	24°C	4°C	24°C		4°C	24°C	4°C	24°C		
S (days)	0	ÇG1	11.11 cd	11.11 cd	**	**	9.26 a	**	**	**	**	1.85 b	**	**	**	**	0.00 b	3.70 a
		ÇG2	22.22 bc	22.22 bc	**	**		**	**	**	**		**	**	**	**		
		ÇG3	**	**	**	**		**	**	**	**		**	**	**	**		
		ÇG4	33.33 ab	33.33 ab	**	**		**	**	**	**		**	**	**	**		
		ÇG5	44.44 a	44.44 a	**	**		**	**	**	**		**	**	**	**		
		ÇG6	33.33 ab	33.33 ab	**	**		**	**	**	**		**	**	**	**		
		ÇG7	**	**	**	**		**	**	**	**		**	**	**	**		
		ÇG8	22.22 bc	22.22 bc	**	**		**	**	**	**		**	**	**	**		
		ÇG9	**	**	**	**		**	**	33.33 ab	33.33 ab		**	**	**	**		
	15	ÇG1	**	**	**	**	0.00 b	**	**	**	**	0.00 b	**	**	**	**	0.00 b	0.00 b
		ÇG2	**	**	**	**		**	**	**	**		**	**	**	**		
		ÇG3	**	**	**	**		**	**	**	**		**	**	**	**		

	ÇG4	**	**	**	**		**	**	**	**		**	**	**	**		
	ÇG5	**	**	**	**		**	**	**	**		**	**	**	**		
	ÇG6	**	**	**	**		**	**	**	**		**	**	**	**		
	ÇG7	**	**	**	**		**	**	**	**		**	**	**	**		
	ÇG8	**	**	**	**		**	**	**	**		**	**	**	**		
	ÇG9	**	**	**	**		**	**	**	**		**	**	**	**		
	ÇG1	**	**	**	**		**	**	**	**		**	**	**	**		
	ÇG2	**	**	**	**		**	**	**	**		**	**	**	**		
	ÇG3	**	**	**	**		**	**	**	**		**	**	**	**		
	ÇG4	**	**	**	**		**	**	**	**		**	**	**	**		
30	ÇG5	**	**	**	**	0.00 b	**	**	**	**	0.00 b	**	**	**	**	0.00 b	0.00 b
	ÇG6	**	**	**	**		**	**	**	**		**	**	**	**		
	ÇG7	**	**	**	**		**	**	**	**		**	**	**	**		
	ÇG8	**	**	**	**		**	**	**	**		**	**	**	**		
	ÇG9	**	**	**	**		**	**	**	**		**	**	**	**		
	ÇG1	**	**	**	**		**	**	**	**		**	**	**	**		
	ÇG2	**	**	**	**		**	**	**	**		**	**	**	**		
	ÇG3	**	**	**	11.11 cd		**	**	**	**		**	**	**	**		
	ÇG4	**	**	**	**		**	**	**	**		**	**	**	**		
60	ÇG5	**	**	**	**	0.62 b	**	**	**	**	0.00 b	**	**	**	**	0.00 b	0.21 b
	ÇG6	**	**	**	**		**	**	**	**		**	**	**	**		
	ÇG7	**	**	**	**		**	**	**	**		**	**	**	**		
	ÇG8	**	**	11. 11 cd	**		**	**	**	**		**	**	**	**		
	ÇG9	**	**	**	**		**	**	**	**		**	**	**	**		
AVG			2.46 a				0.46 b					0.00 b					

*Applications were performed in triplicate and 3 beads were used for each replication. The differences between the values indicated with different letters are significant at the p<0.0.1 level according to the Duncan multiple comparison test. M: Medium code, A: NaAlg dose, C: $CaCl_2$ dose, S: Storage time, T: storage temperature

** 0.00 d

The values of different interactions with storage temperature were also examined within the scope of the study. No statistical difference was determined between the data in the C*T interaction. When the effect of storage periods was examined, day 0 trials were in the first group with both temperature values of 3.70%. Although 0.2% value was obtained for both storage temperatures in the 60-day trials, they were included in the last group along with the other trials (Fig 2).

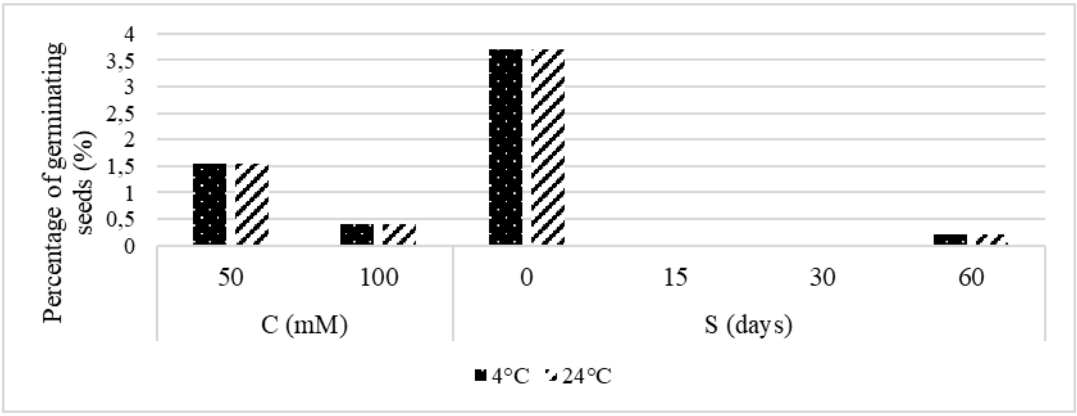

Fig 2. Graph of the percentage (%) data of germinating beads in the interactions of different $CaCl_2$ doses, alginate dose and storage times.

Various observations were made regarding the forms of the beads during the experiments. The beads produced with different amounts of NaAlg and $CaCl_2$ had different hardness and different degrees of opacity. Especially in low $CaCl_2$ and low NaAlg amounts, the beads were extremely soft and elliptical in shape, while harder and rounder shaped beads were formed with increasing $CaCl_2$ amounts (Fig 3). In addition, at high $CaCl_2$ molarity, the beads had a matte appearance, while as the amount decreased, the degree of transparency increased (Fig 4). While transparent beads were softer, as the opacity increased, the hardness of the beads increased. Increasing hardness in the beads had a negative effect on germination. As a result of all the experiments, the germinating seeds were determined and the most suitable coating method, storage condition and duration were determined (Fig 5).

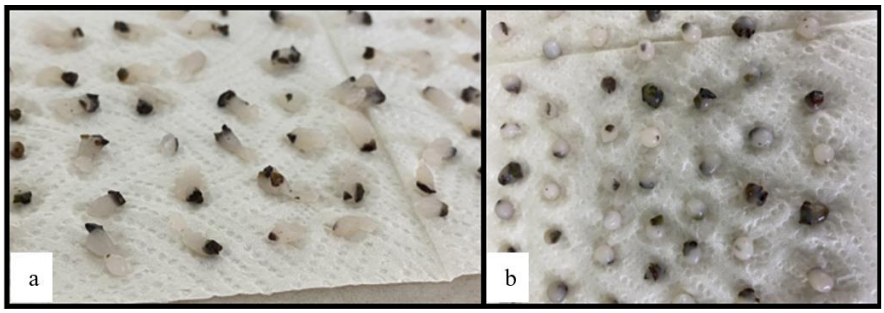

Fig 3. Synthetic seeds in different shapes and hardness states a) 50 mM $CaCl_2$ and 3% Na-Alg b)100 mM $CaCl_2$ and 3% NaAlg.

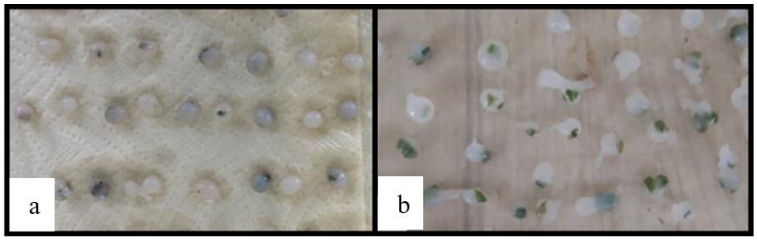

Fig 4. Synthetic seeds with different degrees of opacity a) 100 mM $CaCl_2$ and 3% NaAlg b) 50 mM $CaCl_2$ and 3% NaAlg.

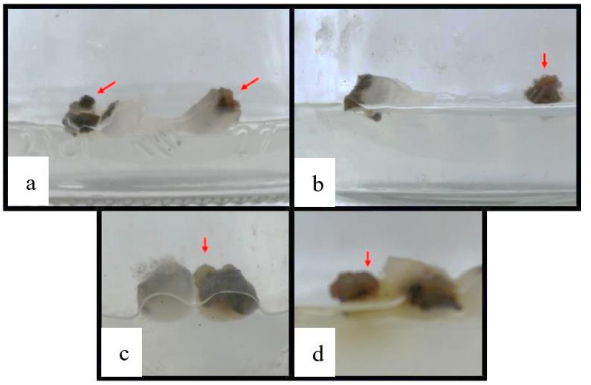

Fig 5. Some synthetic seeds germinating in different nutrient media as a result of storage period a) Seeds coated with 50 mM $CaCl_2$+3% NaAlg and stored for 0 days and germinated in ÇG9 medium, b) Seeds coated with 50 mM $CaCl_2$+3% NaAlg and stored at 4°C for 15 days and germinated in ÇG4 medium, c) Seeds coated with 100 mM $CaCl_2$+3% NaAlg and stored at 4°C for 15 days and germinated in ÇG1 medium, d) Seeds coated with 50 mM $CaCl_2$+3% NaAlg and stored at 4°C for 60 days and germinated in ÇG9 medium (indicated by red arrow).

4. Discussion

Somatic embryos produced in *in vitro* conditions in the tea plant were coated using different doses of NaAlg and $CaCl_2$ and synthetic seeds were obtained. The obtained synthetic seeds were germinated in nutrient media with different compositions after being stored under different conditions and for different periods of time. In this way, a new biotechnological method has been developed that allows it to be used in germplasm preservation in tea plants and facilitates the storage-transport stages.

Contamination is one of the problems frequently encountered in plant tissue culture studies. Contaminations in many cases prevent the growth and development of plants and cause loss of vitality [28]. In many cases, contamination can be seen quickly from the very beginning of the culture during in vitro studies, but in some cases, hidden contamination can become evident in later subcultures [27]. This situation was also seen in the study conducted by us. While no contamination was encountered in somatic embryo studies of the tea plant, yeast contamination was encountered in synthetic seed studies. The higher incidence of contamination, especially in beads stored at 24°C, can be explained by the fact that the ambient temperature provides suitable conditions for the growth of contaminants.

As a result of the study, although germination occurred in long-term storage, the data were not found to be statistically significant. This situation was thought to be related to intense darkening. In the study, a higher average germination rate (2.46%) was observed in the lowest dose alginate applications, while the germination rate decreased with increasing doses. It was determined that the most suitable encapsulation was obtained in the experiments carried out with 3% Na-Alg and 50 mM $CaCl_2$, and in the germinations carried out without storage, the MS nutrient medium containing 3 mg/L BAP and 1 mg/L IBA was the most suitable germination medium. In the experiments, it was determined that an average of 1.333 out of 3 beads remained alive, and the viability rate was determined as 44.44%. Additionally, when $CaCl_2$ doses were compared, the average of 50 mM applications was found to be 1.54%, while this rate decreased to 0.41% in 100 mM trials. Another situation observed in the study is that increasing Na-Alg and $CaCl_2$ doses cause the formation of more round-shaped but harder beads. In encapsulations at lower doses, the shapes of the beads became more elliptical, but softer beads were obtained. Additionally, the opacity of the beads also varied. While the beads coated with higher molarity $CaCl_2$ had a more matte appearance, it was observed that the beads coated with lower molarity were more transparent.

Seran et al. carried out a study on the encapsulation of zygotic embryogenic parts of the tea plant [24]. In the study, it was determined that the most suitable encapsulation was obtained in the combination of 3% NaAlg and 100 mM $CaCl_2$ and the highest germination rate was in MS medium containing 3 mg/L BAP and 0.5 mg/L IBA. Similarly, in the study conducted by us, 3% NaAlg was found to be suitable and the suitable nutrient medium

content for germination was also similar. However, while [24] found the germination rate to be 93%, the germination rate in this study conducted by us was lower. It was thought that this situation was due to the difference in the encapsulated explant. In addition, it was observed in the study that harder and matte beads were obtained in high NaAlg and $CaCl_2$ combinations. [24] carried out experiments on MS nutrient media with and without plant growth regulators for germination and stated that exogenous plant growth regulators were needed for organ formation in tea plantlets. Similarly, in our study, regeneration did not occur in all experiments that did not contain plant growth regulators.

Gantait and Kundu, examined the polymerization abilities of different NaAlg doses in their studies on encapsulation [10]. In the study, experiments were carried out based on the knowledge that the bonding resulting from ion exchange between sodium (Na) and calcium (Ca) affects the form and hardness of synthetic seeds. In experiments using less than 3% NaAlg, it was observed that alginate lost its gelling feature and beads that were too soft and amorphous to hold were obtained. It was determined that the most suitable bead form was obtained in experiments using 3% NaAlg and 75 or 100 mM $CaCl_2$. At these doses, the shape of the beads was determined to be extremely solid, round in shape, light colored and uniform. This is thought to be due to the fact that the coating provides better protection as well as allowing nutrient transport between the explant and the nutrient medium without damaging the bead shape. It has been stated that with increasing doses of NaAlg, opacity increases and germination decreases in direct proportion to the dose. In addition, it was not preferred in the study because it showed toxic effects at high $CaCl_2$ doses. The information obtained from this study is parallel to the information obtained by us.

The genus Camellia is considered one of the most complex plant taxa. This complexity was achieved first by high hybridization and then by polyploidization. The new genetic materials created are preserved by clonal propagation methods. It has been suggested that organogenic calli can be used in micropropagation and long-term storage of tea.

5. Conclusion

Tea plant is an important agricultural plant worldwide with its socio-economic potential. Problems experienced in tea production necessitated the use of biotechnological methods. In our study, synthetic seeds were obtained by encapsulating somatic embryos produced from tea plantlets under *in vitro* conditions, and experiments were carried out to determine the most suitable coating method, storage time, storage condition and germination environment. In the light of the data obtained as a result of the study, the appropriate coating procedure and the appropriate nutrient medium composition for germination were determined. As a result of long storage, germination was observed in synthetic seeds, albeit at a low rate. This data shows that a study has been carried out that will lead to the development of an effective protocol for storage and transportation in the tea plant by applying synthetic seed technology. In addition, the fact that temperature was not determined as an effective factor is an indication that the protocol is economically low-cost. From now on, it is recommended to carry out experiments regarding the coating dose for encapsulation purposes and the use of alternative equipment in addition to the pasteur pipette in coating. In this way, a more practical application method can be used. Additionally, there is a need to carry out experiments by adding NaAlg or $CaCl_2$ or PPM^{TM} to the nutrient medium in order to reduce contamination or browning.

Acknowledgements

This study was supported within the scope of TUBITAK-2211/C National PhD Scholarship Program in the Priority Fields in Science and Technology. This ressearch was derived from Begüm Güler's doctoral thesis.

References

[1] S.S. Chaeikar and K. Falakro, "Effect of colchicine treatment and polyploidy induction on some morphological, physiological and phytochemical characteristics of *Camellia sinensis* cv. Kashef. ". *Journal of Plant Process and Function Iranin Society of Plant Physiology*, vol. 12, no. 54, pp. 203-2018, 2023. doi: http://jispp.iut.ac.ir/article-1-1738-en.html

[2] Direkvand-Moghadam, M.H. Mirjalili, M.N. Padasht Dehkai, H. Hassan Rezadoost. "Aromatic composition, catechins content, and metal elements profiling of forty-three Iranian black tea (*Camellia sinensis* L.) samples." *Journal of Medicinal Plants*, vol. 22, no. 85, pp. 76-88, 2023. doi: 10.61186/jmp.22.85.76

[3] B. Can, B. Güler, and A. Gürel, "Globular embryo regeneration from callus in the tea plant (*Camellia sinensis* [L.] O. Kuntze)." *Indian Journal of Plant Sciences*, vol.11, pp. 62-73. 2022.

[4] Ş.G. İzmirli and S. Gül, "Modeling of current and future distributions of *Camellia sinensis* in Turkiye under climate change." *Theoretical and Applied Climatology*, 2023, https://doi.org/10.1007/s00704-023-04627-6

[5] P. Mangena, "Potential role of somatic embryo-generated synthetic seed production on mass propagation of recalcitrant grain legume crops in Sub Saharan Africa–A review article." *International Journal of Agricultural Technology*. vol. 17, no. 3, pp. 959-976, 2021.

[6] K. Chandra, A. Pandey and P. Kumar, "Sythetic seed-Future prospects in crops improvement." *International Journal of Agriculture Innovations and Research*, vol. 6, no. 4, pp. 2319-1473, 2018.

[7] T.K. Mondal, A. Bhattacharya, A. Sood, and P.S. Ahuja, "Factors affecting induction and cold storage of encapsulated tea (*Camellia sinensis* (L.) O. Kuntze) somatic embryos." *Tea*. vol, 21, pp. 92-100, 2000.

[8] A. Ray and S. Bhattacharya, "Storage and plant regeneration from encapsulated shoot tips of *Rauvolfia serpentina*-An effective way of conservation and mass propagation." *South African Journal of Botany*, vol. 74, pp. 776–779, 2008.

[9] P. Nongdam, "Development of synthetic seed technology in plants and its applications: A Review." *International Journal of Current Science*, vol. 19, no. 4, pp. E 86-101, 2016.

[10] S. Gantait and S. Kundu, "Artificial seed technology for storage and exchange of plant genetic resources." *Advanced Technologies for Crop Improvement and Agricultural Productivity* (Edts: C.P. Malik, S.H. Wani, H. Bhati Kushwaha, R. kaur,), 2017, SBN No.: 978-93-81191-17-0. 2017.

[11] A.A. Qahtan, E.M. Abdel-Salam, A.A. Alatar, Q.C. Wang and M. Faisal, "An introduction to synthetic seeds: production, techniques, and applications (Synthetic Seeds: Germplasm Regeneration, Preservation and Prospects)". Edts: M. Faisal, A.A. Alatar, 2019.Springer. ISBN 978-3-030-24630-3

[12] P. Sharma, B. Roy, M. Roy, and G.S. Sundarrao, "Synthetic seed technology in horticultural crops for conservation and utilisation of germplasm." *International Journal of Agriculture Innovations and Research*, vol. 9, no. 3, pp. 2319-1473, 2020.

[13] M.Ş. Özdemir, "Bazı colchium türlerinde somatik embriyogenesis, histolojik analizler ve sentetik tohum üretiminin araştırılması," Çukurova Üniversitesi Fen Bilimleri Enstitüsü Bahçe Bitkileri Anabilim Dalı. Yüksek Lisans Tezi (Danışman: Prof. Dr. Yeşim Yalçın Mendi). 2021.

[14] V.A. Bapat, "Synthetic seeds: A novel concept in seed biotechnology." *Bhabha Atomic Research Centre Newsletter* vol. 200, pp. 7–111, 2000.

[15] E. Khor and C.S. Loh, "Artificial seeds. In: Applications of Cell Immobilisation Biotechnology" Eds. V. Nedović and R.Willaert, *Focus on Biotechnology*, vol. 8B, 2005.Springer, Dordrecht. https://doi.org/10.1007/1-4020-3363-X_31.

[16] K. Redenbangh, J.A. Fujii, and D. Slade, "Hydrated coatings for synthetic seeds. In: Synseeds" Edt. K. Redenbaugh (CRC Press, Boca Raton). pp. 35-46, 1993.

[17] D.S. Nair and B.R. Reghunanth, "Effective use of encapsulation dehydration technique in cryopreserving somatic embryos of butt erfly pea (*Clitoria ternatea* L.)." *Journal of Herbs, Spices & Medicinal Plants*, vol. 13, pp. 83–95, 2007.

[18] D.S. Nair and B.R. Reghunanth, "Cryoconservation and regeneration of axillary shoot meristems of *Indigofera tinctoria* (L.) by encapsulation–dehydration technique." *In Vitro Cellular & Developmental Biology*, vol. 45, pp. 565-573, 2009.

[19] A. Saxena, M. Shukla and P. Saxena, "Synthetic Seeds: Relevance to Endangered Germplasm Conservation *In Vitro* (Synthetic Seeds:Germplasm Regeneration, Preservation and Prospects)" Edts: M. Faisal and A.A. Alatar, 2019. Springer. ISBN 978-3-030-24630-3.

[20] R. Mohanraj, R. Ananthan and V.N. Bai, "Production and storage of synthetic seeds in *Coelogyne breviscapa* lindl." *Asian Journal of Biotechnology*, pp. 124-128, 2009.

[21] M.M. Lulsdorf, T.E. Tautorus, S.I. Kikcio, T.D. Bethuni and D.I. Dunstan, "Germination of encapsulated embryos of interior spruce (*Picea glauca* engelmanni complex) and black spruce (*Picea mariana* Mill.)". *Plant Cell Reports*, vol. 12, pp. 385-389, 1993.

[22] M.M.M. Micheli and A. Standardi, "Encapsulation of *in vitro* proliferated buds of olive." *Advances in Horticultural Science*, vol. 12, pp. 163-168, 1998.

[23] B. Yücesan,"Synseed: A new trend in seed technology." In:Synthetic Seeds. Edts. M. Faisal and A. Alatar, Springer, Cham. 2019 https://doi.org/10.1007/978-3-030-24631-0_3

[24] T.H. Seran, K. Hırımburagama, and M.T.K. Gunasekare, "Encapsulation of embryonic axes of *Camellia sinensis* (L.) O. Kuntze (tea) and subsequent *in vitro* germination." *Journal of Horticultural Science & Biotechnology*, vol. 80, no. 1, pp. 154–158, 2005.

[25] T. Murashige, and F. Skoog, "A revised medium for rapid growth and bioassay with tobacco tissue cultures." *Physiologia Plantarum*, vol. 15, pp. 473-497, 1962.

[26] N. Onishi, Y. Sakamoto and T. Hirosawa "Synthetic seeds as an application of mass production of somatic embryos". *Plant Cell, Tissue and Organ Culture* vol. 39, pp. 137-145, 1994.

[27] B. Güler, Çay (*Camellia sinensis* [L.] O. Kuntze) bitkisinde geçici daldırma sistemine dayalı biyoreaktörler aracılığıyla sentetik tohumların üretilmesi. Ege Üniversitesi Fen Bilimleri Enstitüsü Biyomühendislik Anabilim Dalı Doktora Tezi (Danışman: Prof. Dr. Aynur Gürel). 2022.

[28] M.B. Pithiye, S.K. Sharma, M. Sharma, M., Sharma and N. Kotwal "Advancement and Challanges in Plant Tissue Culture: A Comprehensive Overview". *Journal of Plant Biota*, vol. 1, no. 1, pp. 12-16, 2022. https://doi.org/10.51470/JPB.2022.1.1.12