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In vitro preservation of somatic seeds and nonencapsulated hemp shoot tips†

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Abstract: Synthetic seed technology and cold storage methods provide genetic uniformity, pest and disease-free plants, easy to handle. The aim of this study was to develop protocols for cold storage of nonencapsulated and alginate-capsulated explants of *Cannabis sativa* L. Axillary shoot tips derived from *in vitro* grown plants were used as explants and stored up to 9 months at 4 °C in the dark. Somatic seeds were produced in 3% sodium alginate and Murashige and Skoog (MS) medium salt and stored up to 3 months. After 6 months of cold storage the highest regrowth 45% was recorded for the nonencapsulated explants. Recovery of somatic seeds was 90% under the same storage condition after 3 months. Well-developed, regenerated plants from encapsulated explants were successfully acclimatized.

Keywords: synthetic seeds; cold storage; hemp, *Cannabis sativa* L., *in vitro* culture.

1. Introduction

Synthetic seed technology and cold storage are used for rapid clonal propagation of plants and germplasm preservation. These methods provides genetic uniformity, pest and disease-free plants, easy to handle, and transport. Various explants, such as shoot tips, nodal segments, axillary buds, somatic embryos, as well as other vegetative parts of the plant, can be encapsulated in an artificial hydrogel. Encapsulation of non-embryonic vegetative propagules has been used as a suitable alternative for micropropagation and short-term storage of valuable medicinal plants [1,2]. Synthetic seeds technology has been also used for germplasm conservation and multiplication. The choice of initial explants, encapsulating agent and matrix, addition of growth regulators and nutrients to the capsules, and experimental conditions, substantially influence the success of synthetic seed production, their storage and regeneration [3]. Prepared, encapsulated synthetic seeds can be stored at low temperature. *In vitro* cold storage without regular subcultures allows to rationalize production of nuclear stocks and maintain gene collections [1]. The storage at low temperature reduces the metabolic rate, minimizes the risk of somaclonal variation and prolong storage time. However, low temperature treatment may induce chilling stress, triggering elevated levels of reactive oxygen species (ROS) and cause injury in propagules during storage [4,5].

The cold storage protocols for *Cannabis sativa* L. are scare and limited to drug type chemotypes [5-8]. In this study, we tested industrial hemp genotypes using shoot tips as initial explants. The aim of this study was to develop protocols for cold storage of nonencapsulated shoot tips and for alginate-capsulated somatic seeds. Viability and survival rate under various treatments and time periods up to up to 9 months were verified as an alternative solution for large-scale propagation and germplasm conservation of valuable hemp genotypes.

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2. Materials and Methods

2.1. Plant material and source of explants

In vitro growing hemp plants were used as source of explants (shoot tips). Hemp cultivars Epsilon 68, Globa and hybrid Carmagnola x K290 (marked as 1565) were tested. Stock shoot cultures were grown for 3 weeks in Magenta GA7 boxes containing half-strength (½ MS) medium [9] with 0.5 mg L^{-1} IAA (Indole-3-acetic acid), 3% sucrose and 8.5% agar (BD BACTO™ Agar). Cultures were maintained at 25 ±1°C, under 18/6 h light/dark photoperiod with 80-120 µmol m^2 s^{-1} fluorescent daylight. Shoot tips were removed from the *in vitro* growing plants at the age of 14–21 days and cultured for the next 2 weeks to stimulate the growth of lateral shoots, as described by Wróbel et al. [10]. After 2 weeks shoot tips derived from axillary shoots were cut and used in the experiments.

2.2. Preparation and storage of non-encapsulated explants

Shoot tips (0.5-1.0 cm, Fig. 1a) were excised from axillary shoots and placed vertically in Magenta vessels containing ½ MS medium and 8.5% agar. Explants (30 per vessel) were stored at 4 °C in the laboratory fridge in dark up to 9 months. Every 3 month, 12-15 explants of each accession were set to regrow on the same medium (½ MS + IAA (0.5 mg L^{-1}), 3% sucrose, 8.5% agar) and place in the growth chamber under the same conditions as hemp cultures. Regrowth of explants was recorded after 3 weeks of culture. After this period, survival rate, the percentage of rooted plants, the percentage of callusing explants and the number of roots per shoot were calculated as well as the length of shoots were measured (cm). Experiment was conducted in four replicates each with 12-15 explants.

2.3. Preparation and storage of encapsulated explants

Shoot tips (0.5-1.0 cm) from axillary shoots of the rooted plants (Epsilon 68 and 1565) were excised and fully submerged in 3% alginic acid sodium salt with full-strength MS basal salt medium supplemented with 3% sucrose. Alginic acid coated buds were drop into 75 mM calcium chloride using automatic pipette for 30 min incubation to harden. Synthetic seeds were kept on ½ MS medium in Petri dishes sealed with parafilm. Dishes were stored in the laboratory fridge at 4°C in dark up to 3 months. The same regrowth conditions and medium was used for plant regeneration of somatic seeds. Experiment was conducted in two replicates each with 12-15 explants. All chemicals were purchased from Merck except Bacto Agar (Becton, Dickinsonand Company, USA) and sucrose (POCH S.A., Poland).

2.4. Acclimatization conditions

The regenerated plants (n=30) were removed from the vessels and washed in autoclaved water, then placed in pots with sterilized soil (standard garden soil without additives) under glass covers and grown at 25 ± 1°C (18/6 photoperiod, 80–120 µmol m^2 s^{-1}). After 1 week, the glass covers were replaced with plastic covers and plants were progressively exposed to the environmental humidity and then hardened for 3–4 weeks. After this period, the percentage of well-developed plants was calculated.

3. Results and Discussion

3.1. Storage and recovery of non-encapsulated explants

One of the key factors affecting the success of cold storage is the choice of the initial explants. In this study we used shoot tips excised from axillary shoots of *in vitro* growing plants. The choice of explant was based on preliminary studies that showed better rooting and shoot regeneration of shoot tips explants contrary to nodal explants. The differences in rooting rate of these two type of explants were significant in all tested hemp accessions, e.g. 86% *vs* 30% for hybrid 1565 or 82% vs 49% for Epsilon 68 cultivar. Therefore the shoot

tips were used as initial explants in both experiments. The effect of cold storage duration on recovery of "naked" explants of hemp is presented in Table 1.

Table 1. Effect of cold storage duration on rooting and shoot regeneration of unencapsulated explants of hemp.

Accession	Storage in months	Survival (%)	Rooting Rate (%)	Mean no. of roots per explants (±SD)	Mean shoot length [cm]	Callusing explants (%)
Epsilon 68	3	100.0	85.7	5.00±3.61	3.08±1.72	31.0
	6	58.1	27.9	2.18±0.98	1.55±1.40	41.9
	9	16.7	4.8	0.92±0.17	0.56±0.24	2.4
Carmagnola x K290 (1565)	3	100.0	71.4	4.45±4.38	2.58±1.56	40.5
	6	81.8	45.5	3.48±1.95	1.92±1.59	72.7
	9	52.4	28.6	2.82±1.29	1.02±1.40	11.9
Globa	3	90.7	48.8	2.48±1.63	1.84±1,43	72.1
	6	86.0	44.2	2.30±1.90	1.50±0.76	69.8
	9	78.6	35.7	2.21±1.33	1.66±1.49	73.8

Duration of cold storage as well as the hemp accession had significant impact on explant recovery and plant regeneration. After 6 months of storage 58-86% explants survived and 28%-45.5% of them fully regenerated root system and shoots (Table 1). After 9 months only 5%-36% of explants developed into vigorous plantlets. Moreover, the effect was genotype-dependent. Epsilon 68 explants were the most sensitive to chill stress and showed the poorest recovery rates after 6 and 9 months of storage. Differences between hemp accessions may result from their adaptation to local climatic conditions e.g. French cultivar Epsilon 68 was more sensitive contrary to Ukrainian cultivar Globa. Globa and 1565 accessions showed similar recovery rates (46% and 44%) after 6 months. It should be noted that "naked" explants showed similar recovery rate (43%) was recorded for encapsulated synthetic seeds of drug type cannabis (MX) [8]. However lack cold storage protocols and other studies on hemp makes it difficult to compare.

a) (**b**)

Figure 1. (**a**) Donor plant (1565) with red marked explants (shoot tips); (**b**). Rooted plants (Epsilon 68) originated from somatic seeds (3 months of storage), 5 weeks after germination.

3.2. Storage and recovery of somatic seeds

Studies on encapsulation of *Cannabis sativa* L. explants are limited to a few papers and concern only high yielding drug type genotypes. For encapsulation axillary buds [6] and nodal explants [5,7,8] were used. 60% regrowth rate was noted for synthetic seeds storage at 15°C during 24 weeks [8]. High regrowth rates 70% and 90% were recorded for synthetic seeds from *in vitro* and *in vivo*-derived plants by Zarei et al. [5]. Encapsulated explants (nodal segments) were storage at 6°C for 150 days. They found that addition of acetylsalicylic acid (ASA) to the encapsulation matrix and light conditions during storage significantly improved germination and regrowth rate of synthetic seeds. To the best of our knowledge, no somatic seed protocols for industrial hemp have been developed nor for shoot tips as initial explants. Regrowth of somatic seeds both hemp accessions is presented in Table 2 and Figure 2b.

Table 2. Regrowth rates of somatic seeds after 3 months of cold storage recorded after 3 weeks after germination

Accession	Survival (%)	Rooting Rate (%)	Mean no. of roots per explants (±SD)	Mean shoot length [cm]	Callusing explants (%)
Epsilon 68	90	55.0	2.34±1.19	3.37±3.20	50.0
Carmagnola x K290 (1565)	100.0	90.0	2.98±2.35	1.50±1.12	70.0

After 3 months of storage relatively high regrowth rates (90% and 55%) was recorded for both hemp accessions. It is worth noting that nonencapsulated explants of Epsilon 68 showed better rooting rates (85.7% *vs* 55%), however this is a preliminary study and the experiment is still ongoing. The final results on larger sample of explants will give the decisive answer which form of preservation and storage is optimal for the tested hemp genotypes. Well-rooted plants, regenerated plants from encapsulated explants were successfully acclimatized with 100% survival rate.

4. Conclusions

In this preliminary study two forms of *in vitro* preservation were tested: nonencapsulated shoot tips and encapsulated explants in sodium alginate explants and stored at 4°C. Results of these preliminary studies show that both methods are potentially useful and suitable for hemp germplasm conservation. However, the protocol of cold storage should be optimized and adapted to the tested hemp genotype.

Supplementary Materials: Not applicable.

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References

1. Lisek, A.; Orlikowska, T. (2004) In vitro storage of strawberry and raspberry in calcium alginate beads at 4°C. *Plant Cell Tissue and Organ Culture* **2004**, 78, 167–172
2. Kikowska, M.; Sliwinska, E.; Thiem, B. Micropropagation and Production of Somatic Seeds for Short-Term Storage of the Endangered Species Eryngium alpinum L. *Plants* **2020**, 9, 498. https://doi.org/10.3390/plants9040498
3. Asadi, R.; Abdollahi, M.R., Moosavi, S.S.; Mirzaie-Asl, A. Alginate encapsulation of micro-cuttings in endangered *Satureja khuzistanica* species: a promising method for obtaining genetically stable plants with high rosmarinic acid content. *Plant Cell Tissue and Organ Culture* **2022**, 151, 307–320. https://doi.org/10.1007/s11240-022-02353-x
4. Lee, J.G.; Yi, G.; Choi, J.H.; Lee, E.J. Analyses of targeted/untargeted metabolites and reactive oxygen species of pepper fruits provide insights into seed browning induced by chilling. *Food Chem*. **2020**, 332, 127406.
5. Zarei, A.; Feyissa, B.A.; Davis, B.; Tavakouli Dinani, E. Cannabis Synthetic Seeds: An Alternative Approach for Commercial Scale of Clonal Propagation and Germplasm Conservation. *Plants* **2022**, 11, 3186.mhttps://doi.org/10.3390/plants11233186
6. Lata, H.; Chandra, S.; Khan, I.A.; ElSohly, M.A. Propagation through alginate encapsulation of axillary buds of *Cannabis sativa* L. An important medicinal plant. *Physiol. Mol. Biol. Plants* **2009**, 15, 79–86.
7. Lata, H.; Chandra, S.; Techen, N.; Khan, I.A.; ElSohly, M.A. Molecular analysis of genetic fidelity in *Cannabis sativa* L. plants grown from synthetic (encapsulated) seeds following in vitro storage. *Biotechnol. Lett.* **2011**, 33, 2503–2508.
8. Lata, H.; Chandra, S.; Mehmadic, Z.; Khan, I.A.; ElSohly, M.A. In vitro Germplasm Conservation of High THC Yielding Elite Clones of Cannabis sativa L. under Slow Growth Conditions. *Planta Med.* **2011**, 77, 3.
9. Murashige, T.; Skoog, F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum* **1962**, 15, 473–97.
10. Wróbel, T.; Dreger,M.; Wielgus, K.; Słomski, R. (2022) Modified Nodal Cuttings and Shoot Tips Protocol for Rapid Regeneration of *Cannabis sativa* L. *Journal of Natural Fibers* **2022**, 19, 536-545, DOI: 10.1080/15440478.2020.1748160