

Alginate encapsulation of micro-cuttings in endangered *Satureja khuzistanica* species: a promising method for obtaining genetically stable plants with high rosmarinic acid content

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Abstract

Synthetic seed production is a suitable method for the rapid, mass, and uniform proliferation of *Satureja khuzistanica* Jamzad, a valuable medicinal plant of the Lamiaceae family. In this study, the encapsulation of micro-cuttings of *S. khuzistanica* was investigated using sodium alginate (SA). In order to determine the best conditions for encapsulation in the *S. khuzistanica* plant, different concentrations of SA and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, different types of explant and matrix culture media, different types and concentrations of plant growth regulator, and different cold storage conditions of synthetic seeds were tested. Also, the genetic stability of regenerated plants from synthetic seeds using the ISSR molecular marker as well as the content of rosmarinic acid in synthetic seed derived plants were evaluated in this study. The use of 3% SA and 100 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ was found to be the best composition for gel complexation. Encapsulation of nodal segments with 1/2 MS medium containing 2.5 μM BAP resulted in the highest regrowth percentage (72.22%) and regrowth rate (0.173). Pre-culture of nodal segments in 1/2 MS medium containing 5 μM IBA and 0.2% activated charcoal for 10 days and the use of 2.5 μM IBA in the rooting medium resulted in the highest rooting percentage (88.88%) in synthetic seed-derived plants. The highest regrowth percentage (61.11%) and regrowth rate (0.173) of synthetic seeds were obtained using 0.45 mg/L TDZ in MS liquid medium. The placement of germinated seeds on the coco peat substrate resulted in the highest conversion rate (61.11%) of synthetic seeds to the plant. Also, storage of encapsulated nodal segments at 4°C in MS culture medium, compared to cold storage without using MS medium, resulted in better regrowth of synthetic seeds. The highest regrowth percentage (44.44%) and regrowth rate (0.092) for cold-stored synthetic seeds occurred after two weeks. The genetic stability testing by ISSR molecular marker showed that synthetic seed-derived plantlets were genetically similar to their mother plants. Also, encapsulated nodal segments and shoot tip derived plants significantly enhanced the rosmarinic acid content up to 7.77 times the natural seed derived plants. Genetic restoration programs, short-term storage, and germplasm distribution in *S. khuzistanica* plants could all benefit from the encapsulation regeneration strategy reported here.

Introduction

Satureja khuzistanica Jamzad is an aromatic and valuable medicinal plant of the Lamiaceae family and an endemic species in southern Iran (Jamzad 1994). There are various chemicals such as tannins, sugars, and essential oils in this plant, making it a valuable plant species for human consumption (Hadian et al. 2011). The most important chemical compounds in the essential oil of *S. khuzistanica* are: carvacrol (over 90%), paracymene, limonin, 1,8-cineole, eugenol, myrcene and alphetogen (Farsam et al. 2004). The carvacrol in the essential oil of this plant species has anti-inflammatory, anti-apoptotic, anti-nociceptive, antibacterial, anti-fungal, and anti-oxidant properties (Liolios et al. 2009; Abid et al. 2014; Dai et al. 2016). Also, rosmarinic acid in the extract of this plant shows antimicrobial activity and prevents Alzheimer's disease (Hamaguchi et al. 2009). In general, the medicinal and biological properties of *S. khuzistanica* make it a valuable plant for use in the pharmaceutical and food industries (Farsam et al. 2004; Hosainzadegan and Delfan 2009). Due to the overuse of this wild plant species for commercial

purposes and its low propagation rate in nature, *S. khuzistanica* has been reported as an endangered plant species (Hadian et al. 2017; Khojasteh et al. 2019) and also a highly threatened species in the Red Data Book of Iran (Jali (Jalili and Jamzad 1999)). Over the past years, herbal drugs of this endemic plant have been collected from wild habitats to supply the demands of industries, leading to problems such as the threat of species extinction and the production of heterogeneous plant material. To resolve these concerns, the domestication and cultivation of this plant are emphasized, which aids in germplasm conservation as well as the quality and homogeneity of plant raw materials in this species (Hadian et al. 2011). Plant tissue culture techniques are very useful methods for the production of uniform plants in a short period of time. These systems are also efficient and faster methods for the biotechnological production of some plant secondary metabolites (Khojasteh et al. 2020). Due to the presence of dormancy in *S. khuzistanica* seeds and its low potential for seed production and seed germination, plant tissue culture methods can be used effectively for plant propagation and also secondary metabolite production in this plant species (Ramak et al. 2011). "Artificial seed technology" is one of the important micropropagation methods in plant tissue culture. This technology is the most effective alternative for preserving plant species that generate non-viable seeds and are difficult to reproduce through conventional methods (Daud et al. 2008). This method has the ability to provide genetically identical, virus-free germplasm, as well as ease of transportation, long-term storage, and inexpensive production costs (Ghosh and Sen, 1994). Synthetic seed technology can be a viable alternative conservation approach for endangered rare plants like *S. khuzistanica* that cannot be propagated effectively through traditional methods. In this method, various explants, such as somatic embryos, shoot tips and axillary buds, as well as other vegetative parts of the plant, can be encapsulated in an artificial hydrogel (Redenbaugh et al. 1986). However, encapsulation of somatic embryos is limited to medicinal plants in which the induction of somatic embryogenesis has been proven (Verma et al. 2010). Therefore, encapsulation of non-embryonic vegetative propagules such as shoot tips and nodal segments has been used as a suitable alternative for micropropagation and short-term storage of valuable medicinal plants (Lisek and Orlikowska 2004; Singh et al. 2010; Katouzi et al. 2011; Gantait et al. 2015; Siddique and Bukhari 2018). In addition to the choice of initial explants, several other factors, such as encapsulating agent and matrix, addition of growth regulators and nutrients to the capsules, and also experimental conditions, substantially influence the success of synthetic seed production, their storage and regeneration (Saiprasad 2001; Gantait et al. 2015). On the other hand, as synthetic seeds become more popular for germplasm conservation and multiplication, the genetic stability of stored plant material must be evaluated. Inter-simple sequence repeat (ISSR) is a simple and polymerase chain reaction (PCR) based marker system that is increasingly being used in crop plants and medicinal plants to analyze the genetic stability and instability of in vitro culture derived plants (Williams et al. 1990).

In recent years, some research groups (Sahraro et al. 2014; Sahraro et al. 2016; Fatemi et al. 2020) have developed different plant tissue culture systems, such as callus, cell suspension, and nodal segment cultures in *S. khuzistanica*, for further scaling up and commercial production of RA as a valuable natural antioxidant. However, so far, the micropropagation system through synthetic seed production has not been studied to increase the amount of secondary metabolites, especially RA, in *S. khuzistanica*.

Therefore, the main objective of this research was to develop an efficient system for synthetic seed production of *S. khuzistanica* and their germination into plants for further propagation. In the following, the genetic stability of synthetic seed-derived plants and also the RA content of these plants were investigated.

Materials And Methods

Plant material preparation

Multiple shoot cultures of *S. khuzistanica* were established by culturing the nodal shoot explants obtained from greenhouse-grown plants. Suitable nodal segments (each segment containing one node) were cut from plants at the reproductive stage and transferred to the laboratory. The nodal segments were washed for 10 minutes in running tap water, surface sterilized for 5 minutes in 1.5 % (v/v) sodium hypochlorite, and rinsed three times in sterile distilled water for 5 minutes. As described by Fatemi et al. (2019 and 2020), the nodal segments were cultured in solid MS medium containing 2 mg/L 6-benzylaminopurine (BAP), 3 % (w/v) sucrose, and 0.2 % (w/v) polyvinylpyrrolidone (PVP). The plants were grown under *in vitro* conditions in a growth chamber (24 ± 2 °C, 16 h light/8 h dark) at Bu-Ali Sina University, Hamedan, Iran. The cultured nodes were incubated in the growth chamber and sub-cultured every 3 weeks. The micro-cuttings, viz., apical tips and nodal segments (3 mm) of *in vitro*-maintained shoots, were excised aseptically from four-week cultures, and these micro-cuttings were used for encapsulation.

Effect of encapsulation matrix

Different concentrations of SA (2, 3, 4, and 5 %, w/v) were prepared for encapsulation using liquid MS media containing 0.2 % PVP, 3 mg/L BAP, 0.25 mg/L IBA, and 3% sucrose. Using distilled water, several $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ solutions (25, 50, 75, and 100 mM) were produced for complexation. Both the gel matrix and the complexing agent were sterilized by autoclaving at 121°C for 20 min after adjusting the pH to 5.8. Encapsulation was accomplished by mixing the nodal segments or shoot tips into the SA solution and dropping them into the calcium chloride solution. The droplets of gel matrix, each containing a single nodal segment or shoot tip, were dropped in the complexing agent and allowed for polymerization and the formation of capsules for 20 min. The alginate beads were then collected, rinsed with sterile water, and transferred to sterile filter paper for 5 min under laminar airflow to remove excess water, before being placed in Petri plates with MS culture media without plant growth regulator for regrowth. As previously stated, the MS culture medium was solidified with 0.8 % agar and autoclaved to sterilize it. All the developed cultures were maintained in a growth chamber at 24 ± 2 °C with a 16 h light/8 h dark photoperiod for regrowth.

Effect of MS culture medium strength, explant type, and concentration of BAP in gel matrix on regrowth

In this experiment, for the encapsulation of two different explant types (nodal segment and shoot tip), a 3 % SA solution prepared in MS culture medium (half and full strength) supplemented with 3 % sucrose,

and different concentrations of BAP (0, 1, 2.5, 5 and 10 μM), were used as matrix agents. Also, 100 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ was prepared in distilled water and used as a complexing agent.

The effect of different concentrations of TDZ in gel matrix and type of culture medium on regrowth

In this experiment, nodal segment explants were encapsulated with a 3 % SA and 100 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ combination in the 1/2 MS culture medium containing different concentrations of thidiazuron (0, 0.1, 0.25, 0.45, and 0.5 mg/L) as the gel matrix. Then, some capsules were transferred to Petri dishes containing solid MS culture medium without any hormones. Other ones were transferred to Erlenmeyer flasks containing 50 ml of liquid MS culture medium (without hormone). The Erlenmeyer flasks were continuously shaken at 120 rpm and kept at a temperature of 24 °C in the growth chamber. The regrowth percentage and rate were calculated after two weeks. Shoot length, number of shoots, and number of nodes were all measured after another four weeks.

The effect of different concentrations of IBA in gel matrix on root induction

In this experiment, nodal segment explants were first pre-cultured on 1/2 MS culture medium supplemented with 0.2 % activated charcoal and 5 μM IBA for 10 days. After 10 days, these explants were encapsulated with a 3 % SA solution prepared in a half-strength MS culture medium containing 2.5 μM BAP and different concentrations of IBA (0, 2.5, 5, and 10 μM) in the gel matrix. A complexing agent of 100 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ was also utilized in this experiment. Two-week-old germinated capsules were put on a planting substrate of coco peat and peat moss (1:1) and generated roots, after two months of encapsulation. The root induction efficiency was assessed by counting the number of roots, root percentage, and root length.

The effect of cold storage periods and conditions on regrowth traits of encapsulated nodal segments

To assess the effect of cold storage on regrowth traits, encapsulated nodal segments were transferred to two glass jars containing solid MS culture medium and without MS culture medium. The samples were then stored in a refrigerator at 4 °C for various time periods (0, 2, 4, 8, and 12 weeks). After each storage period, encapsulated nodal segments were transferred to a hormone-free MS culture medium for regrowth. After 2 weeks, the regrowth percentage and regrowth rate of capsules were calculated for each treatment.

Effect of different planting substrates on plant conversion

Various planting substrates, including MS culture medium without growth regulators, coco peat, perlite, sand, and soil, were assessed for the conversion of encapsulated nodal segments into whole plantlets. Some of the capsules were directly transferred to the sterilized planting substrates immediately after encapsulation. Other capsules were first placed on the free hormone solid MS medium for two weeks, and then the germinated capsules were transferred to the sterilized planting substrates. The planting substrates were irrigated with quarter strength MS culture medium (1/4 MS). After 4 weeks, different traits

such as the percentage of plant conversion, shoot length, stem diameter, and the number of leaves per plant were measured.

Evaluation of genetic uniformity of synthetic seeds derived plants using ISSR molecular marker

Total genomic DNA was extracted using the CTAB extraction method (Doyle and Doyle 1990) from the synthetic seed-derived plants and the mother plant. The quantification of DNA was done using a spectrophotometer (NanoDrop 2000, Thermo Scientific, USA). The DNA samples were diluted to $50 \text{ ng}\mu\text{l}^{-1}$ with TE (Tris–EDTA) buffer before use and stored at $4 \text{ }^{\circ}\text{C}$ for the ISSR experiment. In ISSR analysis, six primers were used for the genetic stability assessment of nine synthetic seed-derived plants compared to their mother plant. The PCR reactions were performed in a $20 \text{ }\mu\text{l}$ volume containing $6 \text{ }\mu\text{L}$ H₂O, $10 \text{ }\mu\text{L}$ master solution (Taq 2X, 2 mM MgCl₂), $2 \text{ }\mu\text{L}$ primers, and $2 \text{ }\mu\text{L}$ template DNA. The PCR reactions were done with a thermal cycler (MJMini Bio-Rad, USA) using a single primer in each reaction. The PCR amplification was adjusted as an initial denaturation at $94 \text{ }^{\circ}\text{C}$ for 5 min, followed by 35 cycles of PCR reaction consisting of denaturation at $94 \text{ }^{\circ}\text{C}$ for 1 min, primer annealing at a specified temperature for each primer for 30 s, and an elongation stage at $72 \text{ }^{\circ}\text{C}$ for 30 s. A final extension at $72 \text{ }^{\circ}\text{C}$ for 7 min was also done. The resolution of the PCR products by ISSR primers was studied by electrophoresis (1% agarose gel for 100 min in 1X TBE buffer and stained with ethidium bromide). The gel documentation system (DigiDoc H110) was used for photography.

HPLC analysis

In this experiment, the rosmarinic acid content of two types of plants derived from the encapsulation of nodal segments and shoot tips was compared to that of natural seed-derived plants. Regenerated plants from synthetic seeds made with 3 % SA and 100 mM CaCl₂·2H₂O in a half-strength MS culture medium supplemented with 3 % sucrose and $2.5 \text{ }\mu\text{M}$ BAP were used for HPLC analysis. HPLC analysis was done according to the protocol described by Fatemi et al. (2019 and 2020). The leaves of *S. khuzistanica* plants derived from natural seeds, encapsulated nodal segments, and encapsulated shoot tips were harvested and dried in a dark condition at room temperature. The samples (1000 mg) were grinded and suspended in 250 ml Erlenmeyer flasks containing 40 ml methanol/water (80/20 v/v), and then transferred to darkness for 2 days. The mixtures were continuously shaken at 80 rpm for 5 h, followed by sonication for 30 min. Finally, the homogenate was filtered through Whatman paper (No. 1) and the filtered solution was evaporated at 50°C using a rotary evaporator (Heidolph, Germany). The residues were dried, and the hydroalcoholic extract was stored in darkness. Different concentrations of rosmarinic acid standard were prepared in 1 mL of methanol/water (50/50 v/v), ranging from 1 to 200 mg/L. Injections derived peak areas were used to calculate the calibration curve. The Spherisorb ODS-2 (5 mg/L) reversed phase $4.6 \text{ mm} \times 250 \text{ mm}$ was used as the HPLC column. Elution was done at a flow rate of 1.0 ml/min at 25°C and detection at 333 nm. The injection volume was $20 \text{ }\mu\text{L}$. Two mobile phases, A (H₂O) and B (methanol), were used. The solvent composition of the gradient was at low pressure, 75 % A and 25 % B for the first 5 min, followed by 50 % A and 50 % B for the next 10 min, and finally 100 % B for an additional 15 min. Each extract (2 mg) was dissolved in 1 mL of methanol/water (50/50 v/v) and

filtered through a 0.45 mm filter. The chromatography peak of rosmarinic acid was confirmed according to the retention time of the reference standard (Fig. S1A). Using Agilent ChemStation software, the quantitative analysis was carried out using external standardization by measuring the peak areas.

Data analysis

All experiments were conducted as factorials based on completely randomized designs with 3 replications. Each Petri dish containing six capsules (synthetic seeds) was considered one replication. All data were subjected to analysis of variance (ANOVA) using SPSS software (version 16) and significant differences between the means were assessed by Duncan's multiple range test (Duncan 1955) at $P \leq 0.05$. Before being analyzed, percentage data was square root transformed ($\sqrt{x + 0.5}$).

Results

In the present study, after establishment of in vitro shoot cultures of *S. khuzistanica* plants (Fig. 1A), the 3 mm micro-cuttings, viz. nodal segments (Fig. 1B) and apical tips (Fig. 1C) were used to produce synthetic seeds. Uniform beads in size and shape were produced when the 3 % (w/v) SA solution was exposed to 100 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ solution for 20 min (Fig. 1D). The regrowth of synthetic seeds occurred after 2 weeks from initial encapsulation (Fig. 1E and 1F). Germinated synthetic seeds produced roots after 2 months of transfer to a mixture of coco peat and peat moss (1:1) as a planting substrate (Fig. 1G). The two-week old, germinated synthetic seeds converted into whole plants after four weeks from transferring to the planting substrates (Fig. 1H and 1I).

Effect of encapsulation matrix on synthetic seed regrowth properties

In this experiment, nodal segments were encapsulated using different concentrations of SA and calcium chloride. The combination of 2 % SA with 100 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ resulted in capsules with the highest regrowth percentage (88.88 %) and regrowth rate (0.22) (Fig. 2A and 2B). However, using 2 % SA produced soft and fragile beads that were too difficult to handle. Application of 3 % SA with 100 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ was the optimal combination for regrowth percentage (72.22 %) and regrowth rate (0.18) of synthetic seeds (Fig. 2A and 2B), creating uniform, easy-to-handle, firm, and clear beads. Due to the bead hardness and delay in shoot emergence, the regrowth percentage and regrowth rate significantly decreased at higher concentrations (4 and 5 %) of SA (Fig. 2A and 2B).

Effect of MS culture medium strength, explant type, and concentration of BAP on regrowth properties of synthetic seeds

In this experiment, we evaluated the influence of MS culture medium salt strength, explant type, and BAP concentration in the gel matrix on the regrowth properties of synthetic seeds. The maximum regrowth percentage (72.22 %) and regrowth rate (0.173) were obtained when nodal segments were encapsulated with a half-strength MS culture medium containing 2.5 μM BAP (Table 1). Use of full-strength MS culture medium containing 5 μM BAP and also half-strength MS culture medium containing 2.5 μM BAP in the

gel matrix of the encapsulated shoot tips resulted in the highest amounts of shoot length (1.93 cm) (Table 1). Encapsulation of shoot tips with a half-strength MS culture medium containing 10 and 2.5 μM BAP produced the maximum number of shoots compared to other treatments (1.83 and 1.78 shoots, respectively). The maximum number of nodes (3.19 nodes) was obtained by using the half-strength MS culture medium and 2.5 μM BAP in the gel matrix of the encapsulated shoot tips (Table 1).

Effect of TDZ and type of culture medium on regrowth properties of encapsulated nodal segments

The results of this experiment showed that the use of 0.45 mg/L TDZ in the gel matrix of the encapsulated nodal segments and transferring them to the liquid half-strength MS culture medium resulted in the highest regrowth percentage (61.11 %) and regrowth rate (0.173) compared to other treatments (Table 2). The use of a solid culture medium for regrowth of the synthetic seeds supplemented with 0.5 mg/L TDZ and also a liquid culture medium for regrowth of synthetic seeds containing 0.45 mg/L TDZ produced the longest shoots (2.75 and 2.68 cm, respectively) in comparison with other treatments (Table 2). The maximum number of shoots (2 shoots) was obtained from synthetic seeds containing 0.1 mg/L TDZ in the liquid culture medium (Table 2). Also, regrowth of synthetic seeds in both solid and liquid culture mediums resulted in the highest numbers of nodes (4.5 nodes), when higher concentrations of TDZ (0.5 mg/L and 0.45 mg/L, respectively) were used in their gel matrix (Table 2).

The effect of IBA on the rooting of encapsulated nodal segments

In this experiment, the use of different concentrations of IBA in the gel matrix of the encapsulated nodal segments was evaluated for root induction. The optimal IBA concentrations for root induction were 2.5 μM and 5 μM , which provided the highest percentages of rooting (88.88 % and 77.77 %, respectively, Fig. 3A). All concentrations of IBA used in this experiment (2.5, 10, and 5 μM) resulted in the highest root length (1.58, 1.50, and 1.33 cm, respectively) compared to the control (Fig. 3B). Also, all of the IBA concentrations (2.5, 5, and 10 μM), produced the maximum root numbers (1, 1, and 0.66, respectively) compared with the control (Fig. 3C).

Effect of low temperature storage on regrowth of encapsulated nodal segments

To evaluate the effect of cold storage on the germination properties of encapsulated nodal segments, the prepared synthetic seeds were stored within glass jars containing MS medium and also without MS medium at 4 °C for different time periods. The maximum amounts of regrowth percentage (72.22 % and 66.67 %, respectively, Fig. 4A) and regrowth rate (0.167 and 0.163, respectively, Fig. 4B) were obtained for control seeds in both conditions, with and without MS culture medium. Among the cold storage times, storage of the encapsulated nodal segments on the MS culture medium for two weeks showed the highest regrowth percentage (44.44 %, Fig. 4A) and regrowth rate (0.092, Fig. 4B) compared to other cold storage times. The regrowth percentage as well as the regrowth rate of the synthetic seeds stored at 4°C gradually decreased with increasing storage weeks in both cold conditions (with and without MS culture

medium), and in the twelfth week, regrowth of the cold stored synthetic seeds was stopped in containers without MS culture medium (Fig 4A and 4B).

Effect of different planting substrates on conversion of encapsulated nodal segments to plantlets

As shown in table 3, the highest percentage of plant conversion (61.11 %), was obtained when synthetic seeds were pre-cultured on the MS culture medium for two weeks before being transferred to coco peat as a planting substrate (indirect transfer). Transfer of germinated synthetic seeds to coco peat after two weeks of pre-culture on MS culture medium and also, direct transfer of them to coco peat (without pre-culture on MS culture medium) resulted in the maximum amounts of shoot length (2.40 and 1.75 cm, respectively) (Table 3). Indirect transfer of synthetic seeds to coco peat and perlite substrates and also direct transfer of them to coco peat substrate resulted in the highest values of stem diameter (1.33, 1.33 and 1.17 mm, respectively) and number of leaves per plant (11.17, 6.42 and 7.33, respectively) (Table 3). Transferred synthetic seeds (indirect and direct) to the soil mixture had the lowest percentage of plant conversion as well as other growth features (Table 3).

Evaluation of genetic stability of synthetic seed derived plants by ISSR molecular marker

The number of bands formed by each primer used in this study, as well as the percentage of polymorphic bands, are listed in Table 4. Each ISSR primer amplified a different number of bands, ranging from 3 (IS1) to 11 (IS19), with an average of 7 bands per ISSR primer. A monomorphic banding pattern (Fig. 5) was observed in all nine synthetic seed-derived plants and their mother plant. According to the findings, each primer's produced bands were monomorphic and similar to the mother plant.

Plants grown from artificial seeds have higher levels of rosmarinic acid than plants grown from natural seeds

HPLC analysis with a standard compound was used to assess the amount of rosmarinic acid in hydro-methanolic extracts of dried samples of encapsulated nodal segments, encapsulated shoot tips, and natural seed derived plants of the same age (Fig. S1 and Fig. 6). Figure 6 shows that the content of rosmarinic acid in leaves of encapsulated nodal segments-derived plants was 78.99 mg/g *dry weight* (DW), which was 7.77 times higher than the content of rosmarinic acid in natural seed-derived plants (10.17 mg/g DW). Also, plants grown from encapsulated shoot tips had a rosmarinic acid content of 77.12 mg/g DW, which was 7.58 times higher than plants grown from natural seed (Fig. 6).

Discussion

The use of SA to encapsulate nodal segments of *S. khuzistanica* was investigated in this report. The effects of SA and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ concentrations, explant type, culture medium salt-strength, various levels of BAP, IBA, and TDZ hormones, methods and times of synthetic seed storage in cold conditions, and type of substrate for seed conversion were all evaluated in order to optimize the conditions for synthetic seed production in *S. khuzistanica* plants. The genetic stability of synthetic seed-derived plantlets was also

studied, as well as the rosmarinic acid content of synthetic seed-derived plants. One of the most important factors in the production and germination of *S. khuzistanica* synthetic seeds is choosing the right concentration of SA and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. In this study, gel complexation with 3% SA and 100 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ results in firm, clear, isodiametric, and easy-to-handle capsules. According to these findings, 3% SA has previously been used to produce firm and well-shaped capsules with the shoot tips of *Solanum nigrum* (Verma et al. 2010), *Glochidion velutinum* (Mallikarjuna et al. 2016), *Rhinacanthus nasutus* (Elangomathavan et al. 2017), and *Withania coagulans* (Rathore and Kheni 2017). Furthermore, we discovered that lower SA (2%) produced fragile beads that were difficult to handle, but greater SA (4–5%) produced hard beads that caused a significant delay in shoot emergence, which is consistent with earlier research findings (Adhikari et al. 2014; Parveen and Shahzad 2014). Also, the use of an appropriate culture medium with a desired hormone content in a synthetic seed matrix is one of the most essential and effective elements in germination efficiency and growth of encapsulated explants. In both explants of *S. khuzistanica*, we found that using half-strength MS culture media led to the highest regrowth percentage, regrowth rate, and other growth features. The best regrowth frequency of synthetic seeds was also recorded using a half-strength MS culture media in *Dendrobium white fairy* (Siew et al. 2014) and *Curcuma amada* Roxb (Raju et al. 2016). In this study, we used two explants, nodal segment and shoot tip, for the production of synthetic seeds in *S. khuzistanica*, and showed that encapsulating the nodal segments in a 1/2 MS culture medium resulted in the highest regrowth percentage and rate. A similar finding was obtained in *Phyllanthus fraternus* by previous researchers (Upadhyay et al. 2014).

We also conducted an experiment to evaluate rooting in *S. khuzistanica* synthetic seeds using different concentrations of IBA in the gel matrix. Pre-culturing nodal segments in root culture medium (1/2 MS media supplemented with 2% activated charcoal and 5 μM IBA for 10 days) before encapsulation increased root induction of the resultant synthetic seeds on a mixture of coco peat and perlite as planting substrate. In consistent with these results, Chand and Singh (2004) reported that pre-culture of explants on 1/2 MS culture medium supplemented with 5 μM IBA for 10 days before encapsulation resulted in simultaneous development of shoot and root. The highest rooting percentage in half-strength (MS) culture medium has also been reported for artificial seed production in *Arnebia euchroma* (Manjkhola et al. 2005). In another study by Saha et al. (2015), microshoots of *Ocimum kilimandscharicum* Guerke, recovered from encapsulated shoot tips, were best rooted on a half-strength MS culture medium containing 1.5 mg/L IBA. Also, the highest rooting frequency was achieved using 15 μM IBA in the gel matrix of encapsulated protocorm-like bodies (PLBs), resulting in the successful acclimatization of the plantlets in *Ansellia africana* (Leopard orchid) (Bhattacharyya et al. 2018). Recently, in the encapsulation of nodal segments of *Allamanda cathartica*, the regenerated shoots rooted on half-strength MS medium containing 0.5 μM IBA (Khanam et al. 2021). As described by previous researchers, IBA is an ideal auxin for in vitro rooting in *Salvia fruticosa* (Arikat et al. 2004), *Salvia nemorosa* (Skala and Wysokinska 2004), and *Salvia hispanica* (Bueno et al. 2010).

In another part of this research, the effect of solid and liquid MS culture media on the regrowth and development of encapsulated nodal segments of *S. khuzistanica* containing different concentrations of

TDZ in their gel matrix was evaluated. The majority of features, such as regrowth percentage, regrowth rate, node and shoot numbers, were all higher when liquid culture medium was utilized instead of solid culture medium, especially when larger concentrations of TDZ were used in the gel matrix. Similarly, the maximum conversion of encapsulated nodal segments into plantlets was obtained in guava (*Psidium guajava* L.) in a liquid MS culture medium (Rai et al. 2008). Also, in synthetic seed production of *Stevia rebaudiana*, when full-strength liquid MS medium was compared with the full strength solid MS medium with or without any growth regulator for the conversion of encapsulated shoot tips and nodal segments, it was found that in liquid medium the frequency of conversion was higher (Ali et al. 2012). Better ventilation and oxygenation of explants, as well as better nutrient absorption from this type of culture medium, may explain the better regrowth results of encapsulated nodal segments in liquid medium. According to the findings of this study, an effective short-term storage protocol for *Ansellia africana* was established using encapsulated protocorm-like bodies (PLBs) induced from seedling nodal segments, with the best response reported on MS medium supplemented with 10 μ M TDZ (Bhattacharyya et al. 2018). Since its first experimental demonstration of cytokinin-like activity by Mok et al. (1982), TDZ has been used to induce PLBs in a variety of plant species and has proven to be superior to traditional purine-based cytokinins like BAP (Asghar et al. 2011).

The cold storage duration and condition significantly influenced the regeneration potential of encapsulated nodal segments of *S. khuzistanica* plants. The regeneration potential of synthetic seeds stored in containers without MS culture medium was significantly decreased after two weeks when compared to control and completely lost after twelve weeks of storage in these conditions (Fig. 4). Artificial seeds stored in MS culture medium-containing containers had a much higher potential for regeneration than the previous synthetic seeds, and their ability to regenerate slightly decreased after two weeks. However, after four weeks, the ability to regenerate in recent synthetic seeds gradually decreased, but these seeds were still able to regenerate after twelve weeks. According to previous research (Danso and Ford-Lloyd 2003), the decrease in regrowth parameters of cold-stored synthetic seeds in this study could be attributed to the alginate matrix inhibiting tissue respiration or a loss of moisture due to partial desiccation during storage. On the other hand, the higher regrowth of stored synthetic seeds in jars containing MS medium could be due to the availability of water and nutrients in MS culture media, which prevents moisture loss in cold-stored synthetic seeds (Redenbaugh et al. 1993). In accordance with the findings of this study, short storage of synthetic seeds at cold conditions (4°C) has previously been reported in various plant species such as *Withania somnifera* (Singh et al. 2006), *Decalepis hamiltonii* (Sharma and Shahzad 2012), *Cassia angustifolia* (Parveen and Shahzad 2014), *Withania coagulans* (Rathore and Kheni 2017), *Solanum tuberosum* (Ghanbarali et al. 2016), *Plectranthus amboinicus* (Arumugam et al. 2019), and *Decalepis salicifolia* (Rodrigues et al. 2020).

The method of synthetic seed conversion and the kind of planting substrate were both crucial in the establishment and growth of *S. khuzistanica* encapsulated nodal segments. Synthetic seeds that were planted directly on planting substrates after encapsulation had a lower conversion rate and developed slower than those that were sown indirectly. The increased percentages of conversion and growth features were seen after the pre-culture of synthetic seeds on MS culture medium for two weeks (indirect

conversion method). The findings of this experiment also showed that coco peat is the optimum planting substrate for converting encapsulated nodal segments. According to these findings, Ghanbarali et al. (2016) discovered that pre-culture of synthetic seeds in basic MS culture medium for two weeks before planting in coco peat resulted in the best regrowth and conversion features among several commercial substrates used to convert potato artificial seeds. Faisal et al. (2012) also discovered that synthetic *Rauvolfia serpentine* seeds in a garden soil mixture had the lowest rate of plant conversion, confirming our findings. When mass propagation of plants is done through synthetic seed production, it is critical to propagate genetically identical plants together and to the mother plant. To achieve this goal, molecular markers, especially ISSR markers, are commonly used. The ISSR-based bands generated in this study were all monomorphic, and no genetic variation was observed between the mother plant and the nine randomly selected synthetic seed-derived plants. Several publications have proposed the use of molecular marker techniques, particularly ISSR, for clonal fidelity of *in vitro* grown plants in various plant systems (Mehrotra et al. 2012; Faisal et al. 2012; Saha et al. 2015; Viehmannova et al. 2016; Kundu et al. 2018; Hatzilazarou et al. 2021). Our findings are consistent with previous researches on the genetic stability of synthetic seed-derived plantlets of *Albizia lebbbeck* (Perveen and Anis 2014), *Malus domestica* (Li et al. 2014), *Limonium hybrid* (Bose et al. 2017), *Erythrina variegata* (Javed et al. 2017), *Sphagneticola calendulacea* (L.) (Kundu et al. 2018), *Sphagneticola cale* (Hatzilazarou et al. 2021).

As indicated in the title, one of the most remarkable achievements of this work is the regeneration of plants with high rosmarinic acid content from encapsulated *S. khuzistanica* explants. Plants derived from encapsulated nodal segments and shoot tips produced significantly more rosmarinic acid in their leaves than plants derived from natural seeds, according to the findings. *In vitro* culture techniques seem to be a promising method to explore sustainable rosmarinic acid production in *S. khuzistanica* under controlled conditions (Sangwan et al. 2001). Establishment of callus and cell suspension cultures and also nodal segment cultures for rosmarinic production in this plant species has been reported by previous researchers (Sahraroo et al. 2014; Sahraroo et al. 2016; Fatemi et al. 2020). However, increased production of rosmarinic acid through the production of synthetic seeds in this medicinal plant and even other medicinal species has not been reported. In a similar experiment, Rodrigues et al. (2020) investigated the level of 2-hydroxy-4-methoxybenzaldehyde (2H4MB) in the roots of *Decalepis salicifolia* artificial seed-derived plants. In contrast to the findings of our work, synthetic seed-derived plants produced 2H4MB in amounts comparable to seed-derived field-grown plants of the same age. Also, consistent with the results of this experiment, many researchers have reported an increase in secondary metabolites, particularly rosmarinic acid, in shoot cultures of some plant species, such as *Thymus lotocephalus* (Gonçalves et al. 2019), *Melissa officinalis* L. (Vanda et al. 2019) and *Eryngium alpinum* L. (Kikowska et al. 2020). As described by other researchers (Khan et al. 2020), the production of bioactive secondary metabolites can be enhanced in medicinal plants with micropropagation techniques. During micropropagation, tiny parts of plants, commonly called explants, excised from different plant species can be micro-propagated under optimized growth conditions of culture media, temperature, and photoperiod (Abbasi et al. 2016). As a result of these *in vitro* cultures, the plant cell growth process and the accelerated but controlled secondary metabolism, the ontogenetic changes occur in the regenerated

plants. These ontogenetic changes can be an important reason for the enhanced biosynthesis of secondary metabolites in micropropagated plants (Khan et al. 2020).

Conclusions

This is the first report of synthetic seed production in *S. khuzistanica* using shoot tip and nodal segment explants. The current methodology offers a promising and cost-effective way for producing large-scale synthetic seeds of this endangered medicinal plant, as well as a simple and efficient approach of producing rosmarinic acid in this plant species. By optimizing the encapsulation conditions, determining the optimal hormones for synthetic seed regrowth and rooting, and using a suitable substrate for plant conversion, we were able to obtain entire plants from synthetic seeds in a plant species whose natural seeds are hard to germinate. Short-term storage of synthetic seeds at 4°C also preserved the regeneration potential of synthetic seeds for up to 30 days. However, if additional research is done on the production and storage of synthetic seeds in this plant, as well as optimizing storage conditions and employing different storage temperatures, long-term storage of these seeds might be possible.

Abbreviations

SA Sodium alginate. BAP 6-Benzylaminopurine. IBA Indole-3-butyric acid. MS Murashige and Skoog. NAA α -Naphthalene acetic acid. TDZ Thidiazuron. HPLC High Performance Liquid Chromatography. RA Rosmarinic acid

Declarations

Author contributions MRA conceived and designed the experiments. RA performed the experiments. MRA and RA analyzed the data and wrote the manuscript. MRA, SSM and AM contributed in preparation of reagents, materials and analysis tools. All authors read and approved the final manuscript.

Declaration of interest statement:

The authors confirm that there are no known conflicts of interest associated with this publication

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Tables

Table 1

Effect of the MS-salt strength, explant type and concentration of BAP in the gel matrix on the regrowth and development traits of *Satureja khuzistanica* plants derived from synthetic seeds after two weeks from encapsulation

MS-salt strength	Explant	BAP (μM)	Regrowth		Shoot length (cm)	Number of shoots	Number of nodes
			%	Rate			
Full	Shoot tip	0	22.22bc	0.045 bc	1.00 abc	0.67 bcd	0.67 de
		1	22.22 bc	0.060 abc	0.42 bc	0.67 bcd	0.67 de
		2.5	33.33abc	0.110 abc	0.77 abc	0.77 bcd	1.64 a-d
		5	33.33abc	0.100 abc	1.93 a	1.00 a-d	2.53 ab
		10	11.11c	0.020 c	0.67 bc	0.67 bcd	0.67 de
	Nodal segment	0	11.11c	0.027 c	0.67 bc	0.67 bcd	0.67 de
		1	27.77 bc	0.068 abc	0.67 bc	1.00 a-d	1.33a-d
		2.5	5.55c	0.017 c	0.33 c	0.33d	0.33 d
		5	11.11 c	0.020 c	0.58 bc	0.50 cd	1.17 bcd
		10	11.11 c	0.033 c	0.33 c	0.33 d	0.33 d
Half	Shoot tip	0	11.11c	0.027 c	0.33 c	0.67 bcd	0.67 de
		1	44.44 abc	0.123abc	1.08 abc	1.00 a-d	2.17 abc
		2.5	44.44 abc	0.073 abc	1.93 a	1.78 a	3.19 a
		5	38.89 abc	0.117 abc	1.22 abc	1.55 ab	2.22abc
		10	44.44 abc	0.103 abc	1.44 ab	1.83 a	2.67 ab
	Nodal segment	0	16.67 bc	0.037 c	0.50 bc	0.67 bcd	1.00 bcd
		1	55.55 ab	0.127abc	0.80 abc	1.00 a-d	1.71 a-d
		2.5	72.22 a	0.173 a	1.14 abc	1.33abc	2.36 abc
		5	44.44abc	0.120 abc	0.86 abc	1.22a-d	1.50 a-d

Each data comes from the observation of 18 encapsulated explants. Different letters indicate significant differences ($P \leq 0.05$) according to Duncan's multiple test

10 55.55 ab 0.150ab 0.89 abc 1.11 a-d 1.78 a-d

Each data comes from the observation of 18 encapsulated explants. Different letters indicate significant differences ($P \leq 0.05$) according to Duncan's multiple test

Table 2

Effect of the type of MS culture medium and different concentrations of TDZ in the gel matrix on regrowth and development properties of encapsulated nodal segments of *Satureja khuzistanica* after two weeks from encapsulation

Type of culture medium	TDZ (mg/L)	Regrowth		Shoot length (cm)	Number of shoots	Number of nodes
		%	Rate			
Solid	0	22.22 b	0.028 d	0.50 c	1.00 bc	1.00 d
	0.1	22.22 b	0.036 d	0.92 bc	1.00 bc	2.50 bcd
	0.25	27.77 b	0.051 cd	0.67 bc	1.00 bc	1.33 d
	0.45	27.77 b	0.058 cd	0.92 bc	1.00 bc	1.83 cd
	0.5	33.33 b	0.057 cd	2.75 a	1.33 bc	4.50 a
Liquid	0	22.22 b	0.054 cd	0.72 bc	0.67 c	1.00 d
	0.1	22.22 b	0.097 bc	2.00 ab	2.00 a	3.00 abc
	0.25	38.89 ab	0.132 ab	1.55 abc	1.17 bc	2.44 bcd
	0.45	61.11 a	0.173 a	2.68 a	1.69 ab	4.50 a
	0.5	44.44 ab	0.110 bc	2.19 ab	1.11 bc	3.66 ab

Each data comes from the observation of 18 encapsulated nodal segments. Different letters indicate significant differences ($P \leq 0.05$) according to Duncan's multiple test

Table 3

Effect of four different substrates and pre-culture on MS culture medium in the conversion of encapsulated nodal segments into plantlets after four weeks

Transfer method	Substrate	Conversion (%)	Shoot length (cm)	Stem diameter (mm)	Number of leaves
Transfer after 2 weeks pre-culture on MS culture medium	Coco peat	61.11 a	2.40 a	1.33 a	11.17 a
	Perlite	44.44 ab	1.21 b	1.33 a	6.42 ab
	Sand	16.66 cd	0.50 c	0.67 abc	3.33 cd
	Soil mixture	5.55 d	0.08 d	0.33 bc	0.67 de
Direct transfer (Without pre-culture on MS culture medium)	Coco peat	33.33 bc	1.75 ab	1.17 a	7.33 ab
	Perlite	27.77 bc	0.50 c	1.00 ab	4.00 bc
	Sand	5.55 d	0.08 d	0.33 bc	0.67 de
	Soil mixture	0.00 d	0.00 d	0.00 c	0.00 e

Each data comes from the observation of 18 different encapsulated nodal segments. Different letters indicate significant differences ($P \leq 0.05$) according to Duncan's multiple test

Table 4

ISSR banding profiles of *Satureja khuzistanica* mother plant and the plants grown from synthetic seeds

SL. No.	Primer code	Primer sequence	Range of amplification (bp)	Annealing temperature (°C)	Total number of bands amplified	Total no. of polymorphic bands
1	IS10	(TCG)5C	150–1300	54	5	0
2	IS7	(ACG)5	250–1500	58	7	0
3	IS19	BDB(TCC)5TC	250–1500	62	11	0
4	IS9	(TCG)5G	250–1500	56	7	0
5	IS25	(GACA)4	200–800	52	7	0
6	IS1	(CT)8RG	250–2000	54	3	0

Figures



Figure 1

Synthetic seeds production of *Satureja khuzistanica* Jamzad by encapsulation of different explants in sodium alginate. (A) The established in vitro shoot cultures of plants in a MS culture medium containing 2 mg/L BAP, 3 % sucrose and 0.2 % PVP, (B) Nodal segment explant (3 mm in size) suitable for encapsulation. (C) Shoot tip explant (3 mm in size) suitable for encapsulation. (D) Nodal segment explants encapsulated in sodium alginate beads. (E and F) Emergence of shoot from encapsulated

explants. (G) Emergence of root from encapsulated explants on 1/2 MS culture medium supplemented with 2.5 μM IBA in gel matrix. (H and I) Conversion of encapsulated nodal segments on Coco peat substrate. Bars B and C: 1 mm; D-G: 1 cm

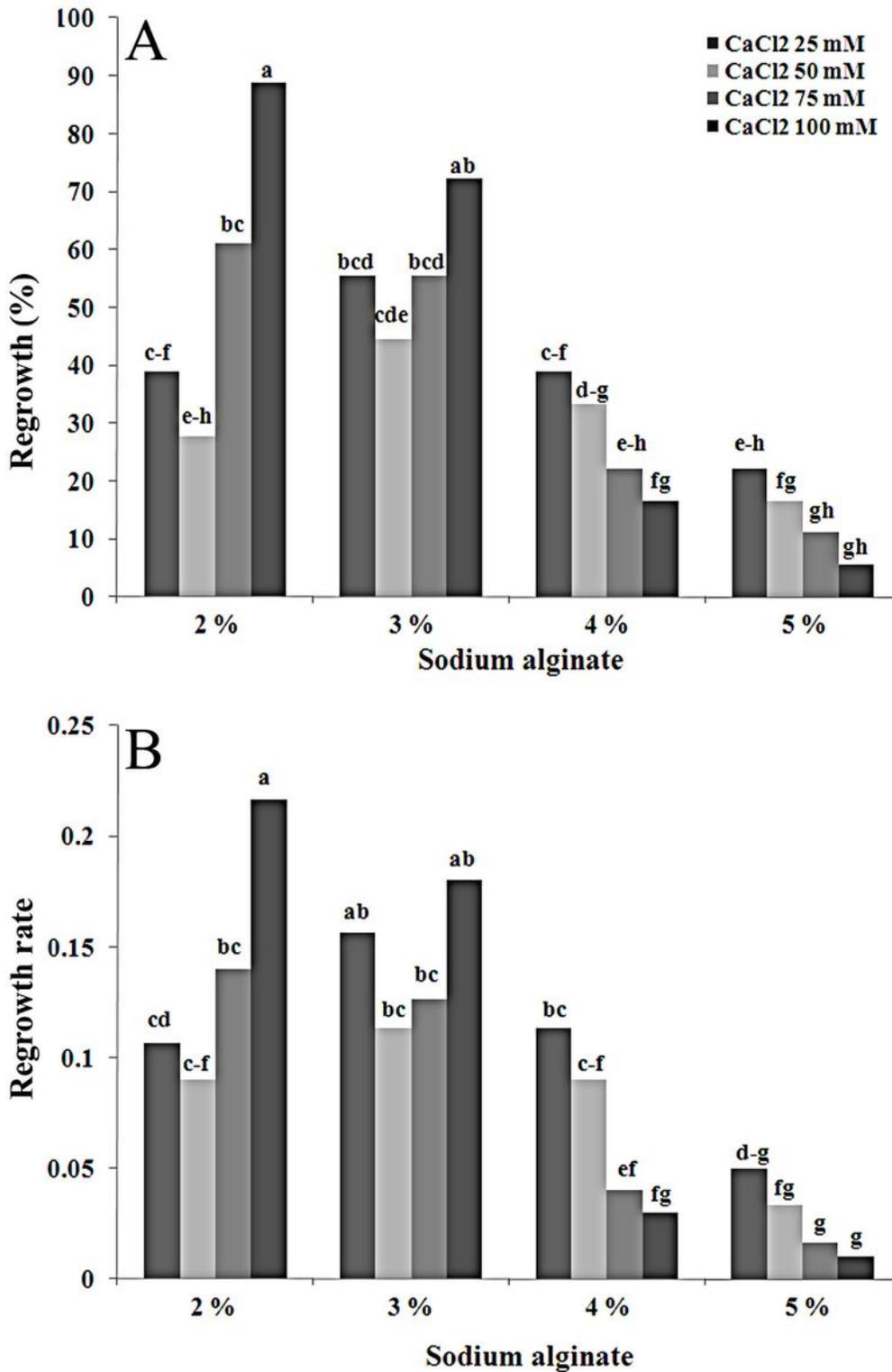


Figure 2

Effect of different concentrations of sodium alginate and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ on (A) regrowth percentage and (B) regrowth rate of encapsulated nodal segments of *Satureja Khuzistanica* Jamzad after two weeks from encapsulation. Each data point comes from the observation of 18 encapsulated nodal segments. Different letters indicate statistically significant differences ($P \leq 0.05$) according to Duncan's multiple test.

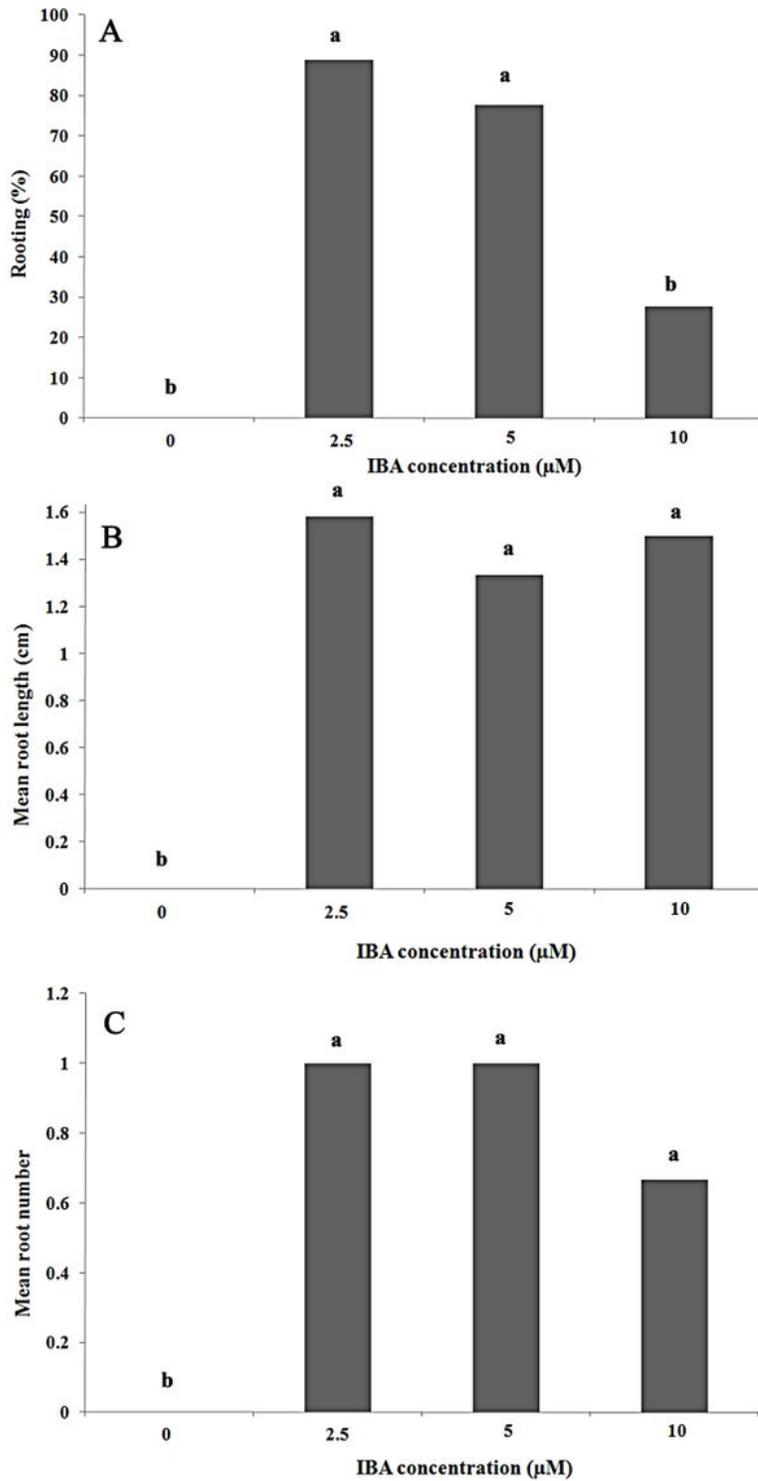


Figure 3

Effect of use of different concentrations of IBA in matrix gel on (A) rooting percentage, (B) root length and (C) root number of encapsulated nodal segments of *Satureja khuzistanica* plants. Each data point comes from the observation of 18 different encapsulated nodal segments. Different letters indicate statistically significant differences ($P \leq 0.05$) according to Duncan's multiple test.

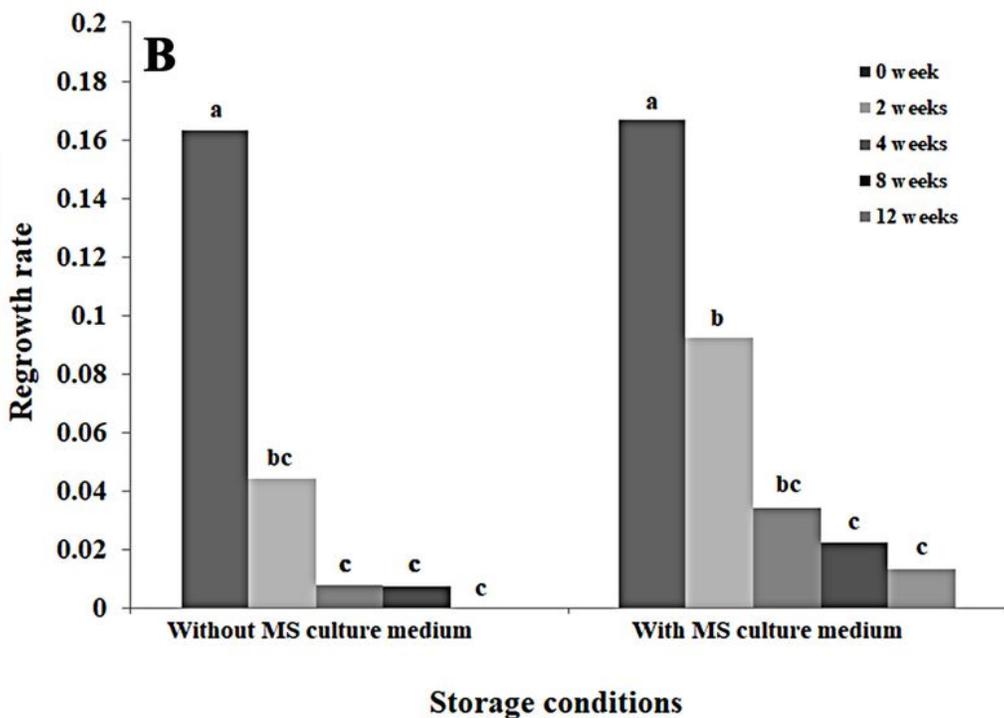
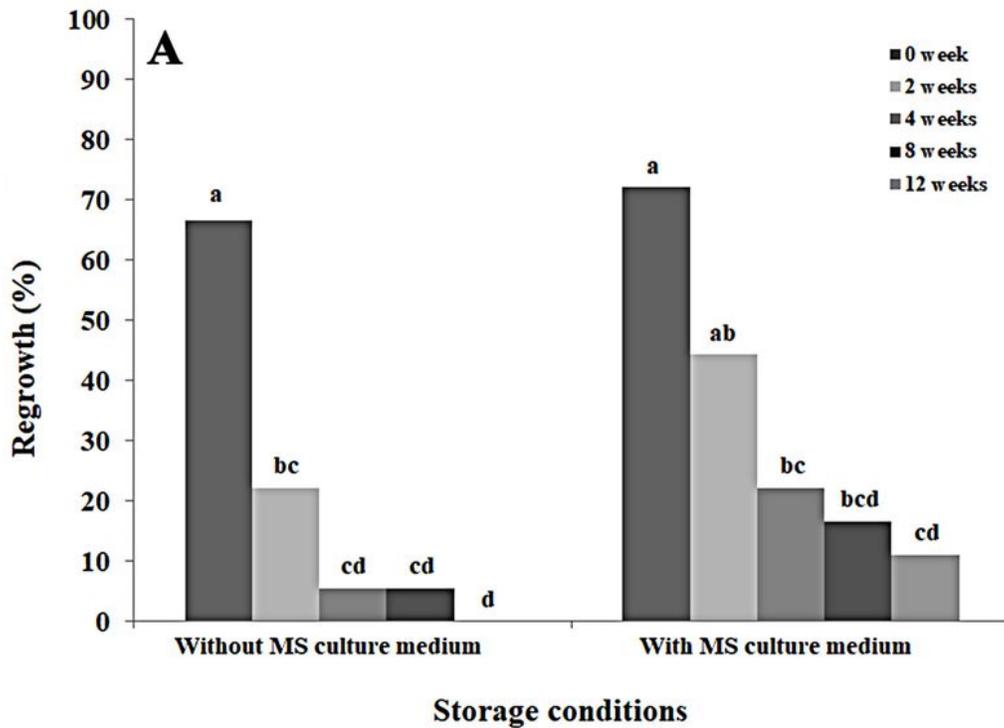


Figure 4

Effect of different cold storage (4°C) periods and conditions on (A) regrowth percentage and (B) regrowth rate of encapsulated nodal segments of *Satureja khuzistanica* plants. Each data point comes from the observation of 18 different encapsulated nodal segments. Different letters indicate statistically significant differences ($P \leq 0.05$) according to Duncan's multiple test.

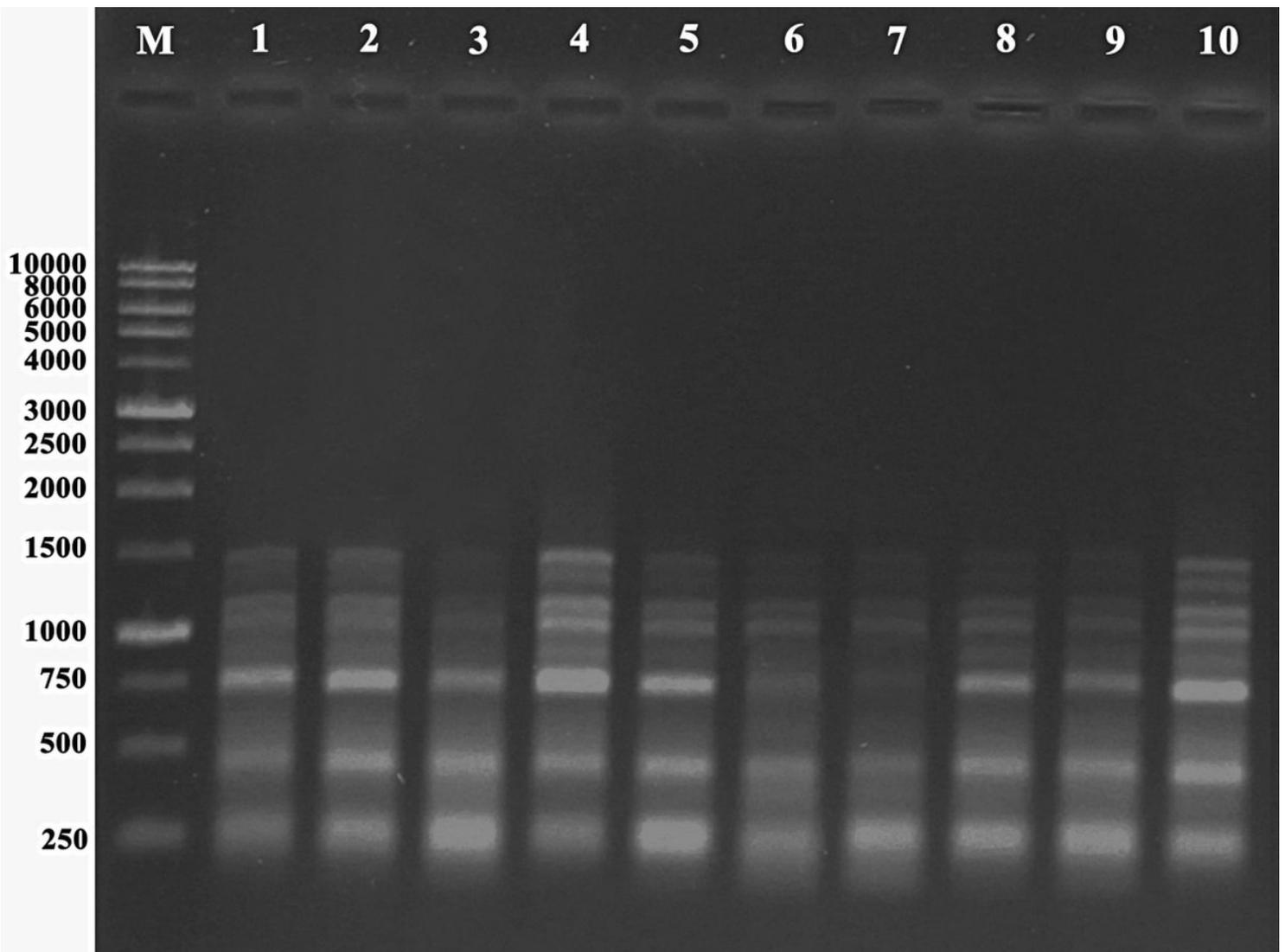


Figure 5

ISSR amplification profiles with primer IS19. M is 1 Kbp DNA Ladder; 1 Mother or control plant; 2–10 plants grown from the synthetic seeds.

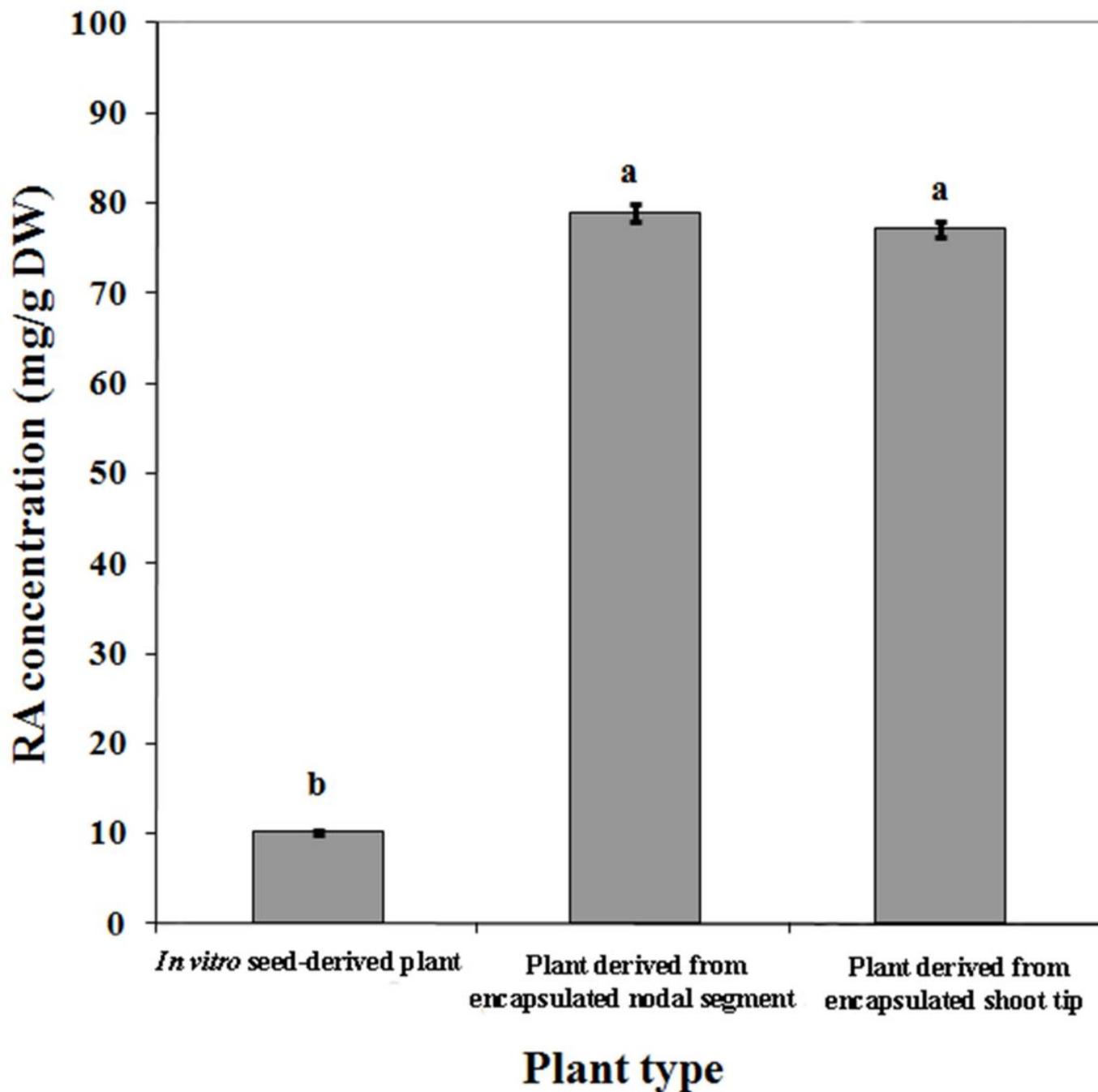


Figure 6

Contents of rosmarinic acid in different *Satureja khuzistanica* plant types derived from natural seeds, encapsulated nodal segments and shoot tip nodal segment. The levels of rosmarinic acid in each sample were analysed by HPLC. Different letters indicate significant differences according to Duncan's multiple range test ($p \leq 0.05$). Bars represent means \pm SD.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryinformationFigureS1.docx](#)