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RESEARCH ARTICLE

Alginate Encapsulation of Shoot Tips and Their Regeneration for Enhanced Mass Propagation and Germplasm Exchange of Genetically Stable *Stevia rebaudiana* Bert.

Tsama Subrahmanyeswari[1] · Saroj Laha[1] · Suchita N. Kamble[2] · Sudhir Singh[2,3] · Somnath Bhattacharyya[1] · Saikat Gantait[1]

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Abstract An accelerated, unique and efficient alginate-encapsulation (synthetic seed)-based genetically true-to-type mass propagation system in stevia (*Stevia rebaudiana* Bert.) was successfully developed. Multiple shoot cultures in stevia were initially established from shoot tip explants cultured on Murashige and Skoog (MS) semi-solid medium supplemented with 0.5 mg/l N^6-benzyladenine. Synthetic seeds were developed from individual shoot tips (obtained from in vitro multiple shoots) using 3% (*w/v*) sodium alginate and 75 mM calcium chloride solutions. In comparison with non-encapsulated in vitro shoot tips, alginate-encapsulated ones (i.e. synthetic seeds) exhibited accelerated germination (in the form of fresh shoot emergence) (~ 2 days), higher shoot proliferation rate and elongation (9.47 shoots with 10.94 mm length, and 64.33 leaves) as well as quicker root initiation (~ 9 days), multiplication and elongation (~ 13 roots with 38 mm length) after 30 days of inoculation in filter paper (M-bridge)-immersed half-strength MS liquid medium without plant growth regulator. Non-encapsulated in vitro shoot tips failed to initiate roots and showed reduced shoot growth. Synthetic seed-regenerated complete plantlets were successfully acclimatized in cocopeat. Clonal fidelity analysis of synthetic seed-regenerated plantlets was performed using ten inter-simple sequence repeats primers that exhibited monomorphic banding pattern, ensuring no genetic variations among the plantlets as well as with their mother plant. The present protocol will be beneficial not only for the smooth exchange of germplasms but also as an alternative approach for accelerated-cum-enhanced in vitro mass propagation of stevia.

Keywords Alginate encapsulation · Germination · Germplasm exchange · ISSR · Mass propagation · M-bridge · Synthetic seed

Abbreviations

BA	N^6-benzyladenine
MS	Murashige and Skoog (1962)
MSL	MS liquid medium
ISSR	Inter-simple sequence repeats
RAPD	Random amplified polymorphic DNA

Introduction

Stevia rebaudiana Bert. (2n = 22), popularly known as "Candy leaf", is indigenously native to Paraguay. It is a perennial small herbaceous short-day plant belonging to the family *Asteraceae* that grows up to 65–80 cm in height. The genus *Stevia* approximately consists of 150–200 species that are extensively cultivated in many parts of Asian countries, viz., Malaysia, Singapore, Taiwan, China, South Korea and Thailand (Gantait et al. 2015). It is widely distributed all around the world, from the USA to Argentina and the Brazilian part, across Mexico and to the South

✉ Saikat Gantait
saikatgantait@yahoo.com

[1] Crop Research Unit (Genetics and Plant Breeding), Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia, West Bengal 741252, India

[2] Plant Biotechnology and Secondary Metabolites Section, Nuclear Agriculture and Biotechnology Division, Bhabha Atomic Research Centre, Mumbai 400 085, India

[3] Homi Bhabha National Institute, Anushaktinagar, Mumbai 400094, India

American Andes and the Central American areas (Gantait et al. 2018).

High demand of this plant as a natural sweetener, ultimately drove for its large-scale cultivation (Das et al. 2005). The production of stevia increased extensively in China, and the chief market is located in Japan. The leaves of this plant contain non-caloric ent-kaurene diterpene glycosides (rebaudioside and stevioside), which are 300 times sweeter than sucrose. It acts as natural sugar and is an alternative to many of the artificially produced sugar substitutes. Traditionally, stevia is propagated via seeds and cuttings, but such practices have major drawbacks, *i.e.* self-incompatibility of seeds, poor seed setting and germination (Gantait et al. 2018). In addition, there is a high chance of genetic variation that ultimately not only may reduce the quantity and quality of pharmaceutically important stevioside content but also delimit the rapid mass propagation of this plant (Das et al. 2011). Since recent past, in many countries, stevia has been familiarized for its ethnopharmacological properties (Gantait et al. 2015). Still, large-scale mass propagation techniques are yet being optimized to increase the quality and quantity of stevioside level in selected elite clones. In such circumstances, alginate-encapsulation-based synthetic seed production is an effective procedure for the storage and exchange of these elite clones.

Synthetic seed production technique acts as an alternative to in vitro mass propagation by reducing the cost of production, useful for short- to mid-term storage of elite germplasms as well as a higher rate of multiplication (Kikowska and Thiem 2011). Among different explants used for encapsulation, shoot tip was found to be best explant for rapid regeneration response in the case of several other medicinal plants (Gantait et al. 2015). In addition, the frequently used basal nutrient medium improves germination and plays a significant role in regeneration of alginate-encapsulated explant (synthetic seed) into complete plantlet. In this context, liquid basal medium is predominately preferred over semi-solid medium since the liquid form significantly provides more nutrients to the explant than solid form of media. Although several media combinations have been widely used for regeneration of synthetic seeds, but to date, there is no report on use of liquid medium provided with filter paper bridge for regeneration of synthetic seeds or mass propagation in stevia. Gantait et al. (2017a,b) reported half-strength Murashige and Skoog liquid (½MSL) basal medium (Murashige and Skoog 1962) provides an optimal level of nutrients for improved germination of the synthetic seeds in case of other medicinal plants like *R. serpentina* and *T. indica*. Faisal et al. (2012) and most recently Gantait et al. (2022) further reported the effectiveness of liquid medium provided with filter paper M-bridge during simultaneous shooting and/or rooting from synthetic seeds of *R. serpentina*. Recently, Catana and Boboc (2021) reported the use of sponge like structure of *Luffa cylindrica* as a supportive material instead of filter paper in the liquid medium for mass propagation of stevia; however, therein an adverse effect was observed during root initiation.

So far, there is no report on usage of filter paper (M-bridge) immersed in ½MSL basal medium for the improvement of germination efficiency of synthetic seeds as well as mass propagation of stevia. The eventual success of any in vitro propagation system lies on effective acclimatization; in this context, merely two reports from Lata et al. (2013) and Shaafi et al. (2021) are available on acclimatization of synthetic seed-derived in vitro regenerants of stevia.

For an effective synthetic seed production with the objective of germplasm storage and exchange, confirmation of genetic integrity is a mandatory step. Yet, there is merely one report confirming the genetic integrity of regenerated synthetic seeds of stevia to date. That is also not comprehensive with fewer primers and resultant bands (loci) (Lata et al. 2016). Inter-simple sequence repeats (ISSR) markers were the most commonly used amid several PCR-based molecular systems to detect the clonal fidelity (Gantait et al. 2017a) because they are cost-effective, simple and easily accessible. As it requires less quantity of DNA and is more convenient, this technique is deemed more advantageous for clonal fidelity screening (Gantait et al. 2017a).

Based on backdrop mentioned above, the present study deals with the establishment of in vitro multiple shoot cultures, development of synthetic seeds from shoot tips, their accelerated germination ensuring enhanced growth and proliferation (required for germplasm exchange), acclimatization of synthetic seed-derived plantlets and evaluation of genetic fidelity of the regenerants using ISSR primers.

Materials and Methods

Plant Material and Establishment of In vitro Multiple Shoot Cultures

Actively emerging shoot tips (2–3 cm in length) from two-months-old stevia plants (Fig. 1A) were used as explants for establishment of in vitro multiple shoot cultures. The explants were sterilized according to the method described by Gantait and Sinniah (2013). The disinfected explants were then briefly dried on blotting paper (Fig. 1B). The explants were trimmed to ~ 1.5 cm before they were inoculated on MS semi-solid medium containing 0.44% (w/v) MS salt (Himedia Laboratories Pvt. Ltd., India), 3%

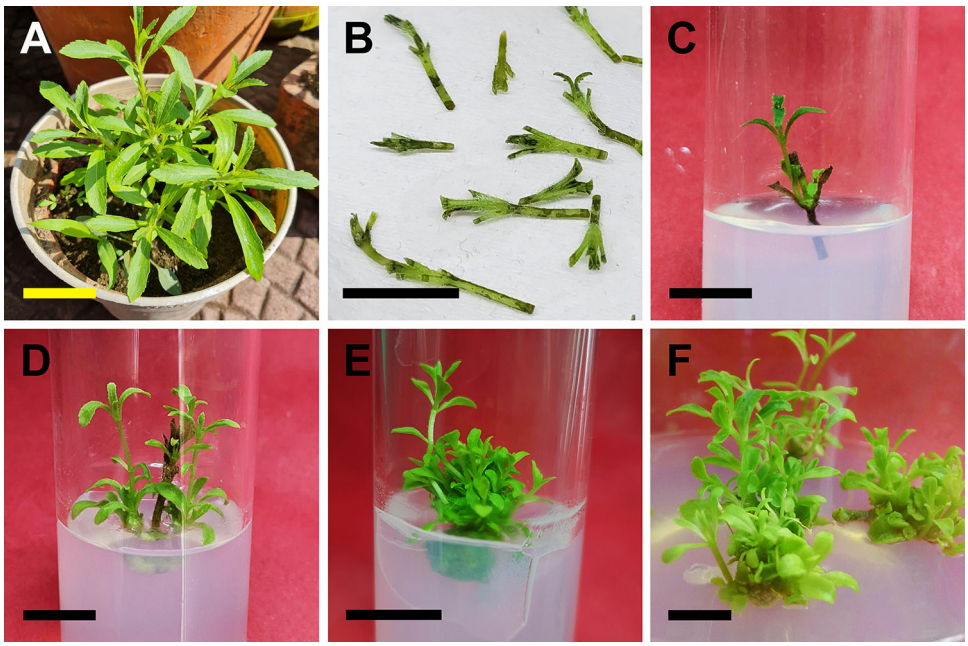

Fig. 1 Establishment of multiple shoot cultures of *Stevia rebaudiana* Bert. **A** Two-month-old ex vitro growing mother plant (source of explants), **B** isolated and surface disinfected shoot tips for inoculation, **C** shoot initiation from shoot tip after 2 weeks of inoculation on Murashige and Skoog semi-solid medium supplemented with 0.5 mg/l N^6-benzyladenine, **D** initiation of multiple shoot after 4 weeks of culture, **E** proliferation and elongation of multiple shoots after 6 weeks of culture, **F** mass multiplication in culture vessels after 8 weeks of culture (Bar = 10 mm)

(w/v) sucrose (Merck Life Sciences Pvt. Ltd., India), 0.7% (w/v) agar (Sisco Research Laboratories Pvt. Ltd., India) supplemented with 0.5 mg/l N^6-benzyladenine (BA) (Fig. 1C). The medium was attuned to pH 5.8 by adding 0.1 N NaOH or 0.1 N HCl before addition of agar and was autoclaved for 20 min at 1.1 kg/cm^2 (121 °C) pressure. After 8 weeks of culture, freshly developed multiple shoots were isolated and shoot tips were encapsulated for the development of synthetic seeds (please refer *'Development of synthetic seeds and assessment of their regeneration'* section). ½MSL medium was used to study regeneration response of encapsulated shoot tips. All the cultures were maintained at 25 ± 1 °C temperature and 60% relative humidity under 16-h photoperiod with 60 µmol/m^2/s photosynthetic photon flux density provided by cool fluorescent tubes (Philips Lifemax, PHILLIPS, India).

Development of Synthetic Seeds and Assessment of Their Regeneration Efficiency

The shoot apices from in vitro shoots of stevia were encapsulated according to Gantait et al. (2017a) using autoclaved solutions of 3% (w/v) sodium alginate (Sisco Research Laboratories Pvt. Ltd., India) dissolved in ½MSL medium and 75 mM of calcium chloride ($CaCl_2.2H_2O$) (Merck Specialties Pvt. Ltd., India). Shoot tips (3–4 mm long size) were isolated from the multiple shoot clumps (Fig. 2A) and plunged in sodium alginate solution for about 10 min (Fig. 2B) inside laminar airflow chamber. Next, a little quantity of sodium alginate, each comprising one shoot tip, was drawn in and then gently dropped in calcium chloride solution under sterile condition or aseptically. Sodium alginate containing the shoot apex was kept immersed as such in calcium chloride solution for 30 min (Fig. 2C) in order to allow complete polymerization that finally lead to the formation of translucent isodiametric beads (i.e. synthetic seeds) (Fig. 2D-E). The synthetic seeds were washed gently with autoclaved water, blotted dry and inoculated on ½MSL medium provided with M-bridge filter paper (ensuring that synthetic seeds were in contact with culture medium) (Fig. 2F) and kept under above-mentioned culture conditions. Data on various parameters such as period required for germination of synthetic seeds (initiation of fresh shoot and root initiation in regenerated shoots) as well as length (mm) of regenerated shoots and roots, and number of leaves per synthetic seed were recorded.

Acclimatization

The well-rooted plantlets regenerated from synthetic seeds were washed thoroughly in running tap water without damaging the roots and transferred to cocopeat substratum for acclimatization. Intermittent spraying of water was done regularly in order to maintain sufficient moisture level. Throughout the entire acclimatization step, an average of (28 ± 2) °C temperature under 12-h photoperiod of 200 µmol/m^2/s photosynthetic photon flux density at 80% relative humidity was maintained for growth and survival of plantlets.

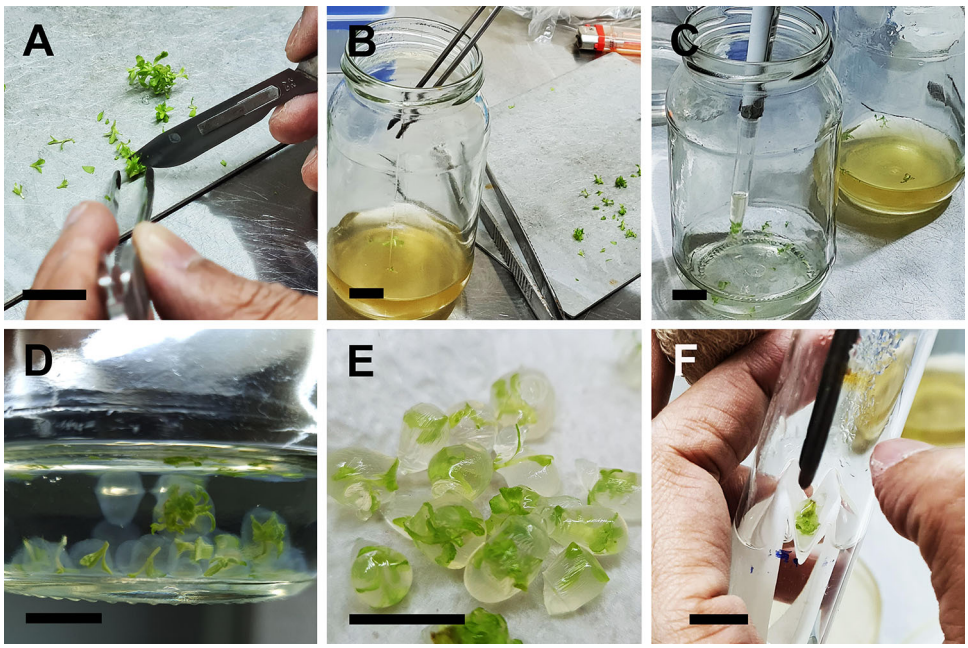

Fig. 2 Development of synthetic seeds of *Stevia rebaudiana* Bert. using in vitro-regenerated shoot tips. **A** isolation of uniform sized shoot tips from multiple shoot clusters, **B** drenching of isolated shoot tips in 3% sodium alginate, **C** dropping of sodium alginate-drenched shoot tips in 75 mM calcium chloride solution, **D** calcium-alginate polymerization of beads, **E** well-developed synthetic seeds, **F** inoculation of synthetic seeds in ½Murashige and Skoog liquid medium supported with filter paper M-bridge (Bar = 10 mm)

Clonal Fidelity Assessment Using Molecular Markers

Detection of the fidelity of plantlets regenerated from synthetic seeds among themselves as well as with their mother plant was carried out with 10 ISSR primers. Genomic DNA was extracted from 6 randomly selected acclimatized plantlets and the source/mother plant using GSure® Plant Mini (DNA extraction) Kit [GCC Biotech (India) Pvt. Ltd., Kolkata]. Polymerase chain reaction (PCR) was carried out in a thermocycler system (Pro-Flex™ Applied Biosystems® by Life Technologies™, Singapore). Each 25 µl PCR reaction contained 12.5 µM PCR master mix, 40 ng of template DNA and 0.1 µM of primer. The PCR programme consisted of initial denaturation step of 5 min at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at (41°–55 °C) (annealing temperature varied according to T_m of primers), extension of 1 min at 72 °C and final extension of 7 min at 72 °C. The PCR products were resolved on 1.5% (w/v) agarose gel stained with ethidium bromide in 1X Tris–borate-EDTA buffer. The size of amplicons was estimated using 100 bp ladder. The gels were then examined under UV light in the UVP® Gel Doc system (Cambridge, UK). The data were noted as '1' for the presence of the band and '0' for the absence of band for each of the synthetic seed-regenerated plantlets and their mother plant. All amplification reactions with ISSR primers were repeated at least three times to check the reproducibility.

Collection of Data and Statistical Analysis

In the present study, the experiments were performed using completely randomized design (CRD). For synthetic seeds germination, 15 replicates (each replica consisted of 5 samples) (with three repeated experiments) were taken to minimize the error. Recorded data was statistically analysed for analysis of variance (ANOVA). Statistical significance was calculated using SPSS (version 17.0, SPSS Inc., Chicago, IL, USA) software. Further, significant variation amid the treatment data (mean ± standard error) was formulated using Tukey's test at $P = 0.05$ level of significance.

Results and Discussion

In vitro Multiple Shoot Cultures

In the present study, multiple shoot cultures of stevia were established from actively growing ex vitro shoot tips cultured on MS semi-solid medium supplemented with 0.5 mg/l BA (Fig. 1A, B). Such low concentration of BA was substantially effective wherein the explants showed initiation of regeneration after 2 weeks (Fig. 1C) and multiple shoot initiation after 4 weeks of inoculation (Fig. 1D). Multiple shoots were further proliferated and elongated within 8 weeks of inoculation (Fig. 1E, F) in the same medium. Promotional effect of BA as the widely used sole cytokinin source proved to be effective in case of stevia as reported earlier (Rafiq et al. 2007; Hassanen and Khalil 2013; Tiwari et al. 2013; Nower 2014; Aziz and Al-

Taweel 2019). Nonetheless, earlier report suggested that higher concentrations of BA resulted in decreasing multiple shoot formation in stevia (Sivaram and Mukundan 2003). It was noteworthy to observe that such a low concentration (0.5 mg/l) of BA proved to be significantly more efficient that its lower (0.25 mg/l) or higher (1 mg/l) concentrations during multiple shoot culture establishment of medicinal plant species like goji berry (*Lycium barbarum* L.) (Karakas 2020).

Development of Synthetic Seeds via Alginate Encapsulation

The development of synthetic seeds from in vitro-regenerated shoot tips using 3% sodium alginate and 75 mM calcium chloride solutions was successfully attained according to the protocol of Gantait et al. (2017a) (Fig. 2A–D). The complexation of gel as well as consistency of capsule depends upon most appropriate exchange of Na^+ and Ca^{2+} ions. The aforementioned encapsulating agent and gelling matrix combination was found to be effective, resulting in isodiametric, firm and translucent beads of uniform shape and size (Fig. 2E). The length of shoot tips $\sim$ 3 mm was proved to be effective wherein this explant size ensured ideal-shaped synthetic seed formation and resulted in quick germination and subsequent high-frequency regeneration than non-encapsulated shoot tips. The effective use of 3% sodium alginate and 75 mM calcium chloride for synthetic seed development was reported in like *Withania somnifera* (L.) Dunal (Singh et al. 2006), *Ocimum basilicum* (Siddique and Anis 2009), *Tuberaria major* (Coelho et al. 2014), and several other herbaceous medicinal plant species (reviewed by Gantait et al. 2015). According to Gantait et al. (2015), 3% sodium alginate and optimum concentration of calcium chloride might have facilitated useful ion exchange between Na^+ and Ca^{2+}, developing translucent, condensed and isodiametric synthetic seeds. Lower or higher concentrations than 3% sodium alginate reported to be inefficient resulting in either asymmetrical and fragile in shape or very hard forms of synthetic seeds, respectively.

Germination of Synthetic Seeds and Mass Propagation

Synthetic seeds (encapsulated shoot tips) and non-encapsulated shoot tips were cultured on ½MSL medium provided with filter paper (M-bridge) according to the procedure optimized by Gantait et al. (2022) to study their regeneration and multiple shoot proliferation response. Encapsulated shoot tips showed accelerated rate of germination (in the form of fresh shoot initiation within $\sim$ 2 days, shoot multiplication and elongation (on an average 9.47 shoots with 10.94 mm length, and 64.33 leaves/synthetic seed) as well as root initiation in shoots ($\sim$ 9 days), multiplication (13.20) and elongation (38.1 mm) at 30 days of inoculation (Fig. 3, 4A–C), and complete plantlet development was observed within 30 days of inoculation (Fig. 4E).

On the other hand, non-encapsulated shoot tips showed delayed shoot initiation ($\sim$ 7 days) and fewer shoots with reduced growth after 30 days of inoculation (Fig. 3, 4F–G) in the same media. Superior performance of encapsulated shoot tips, in terms of growth and development, may be due to better availability of nutrients from the alginate rich ½MSL encapsulation as well as ½MSL medium supported with filter paper (M-bridge) that in turn provided higher surface area for contact of synthetic seeds with the nutrient solution. It was noteworthy to mention that non-encapsulated shoot tips did not produce roots and exhibited regression in shoot initiation because of less availability of nutrients due to absence of alginate encapsulation.

Till date, there is no such report on multiple shoot proliferation in stevia with simultaneous shoot and root development in the same media. Inclusion of filter paper (M-bridge) as support material in MSL medium was found to be effective for the enhancement of rooting response in in vitro shoots obtained from synthetic seeds of *R. serentina.* However, the utilization of substrata (such as filter paper or cotton bed) for complete plantlet regeneration wasn't performed substantially (Faisal et al. 2012; Gantait et al. 2022). In addition to this, Gantait et al. (2017a, b) also reported the positive influence of ½MSL medium for efficient germination and the growth of synthetic seeds in case of *R. serpentina* and *T. indica.* The efficiency of ½MSL medium for improved germination of synthetic seeds was also highlighted by Kundu et al. (2018) in *Sphagneticola calendulacea*; nonetheless, in contrary to the present study (involving sole use of ½MSL medium) they reported the supplementation of α-naphthalene acetic acid and BA in ½MSL medium.

The present study reports for the first time an efficient multiple shoot proliferation protocol with simultaneous shoot and root development (monophasic protocol) from synthetic seeds of stevia cultured on ½MSL medium supported with filter paper (M-bridge).

Acclimatization

The in vitro regenerated plantlets from the synthetic seeds were successfully acclimatized (with 90% survival rate) in cocopeat (Fig. 4H). Emergence of new leaves was observed with no phenotypic variations during their vegetative growth period. According to Subrahmanyeswari et al. (2022), cocopeat provides optimal water retention and enhanced aeration ensuing in ideal nutrient supply for

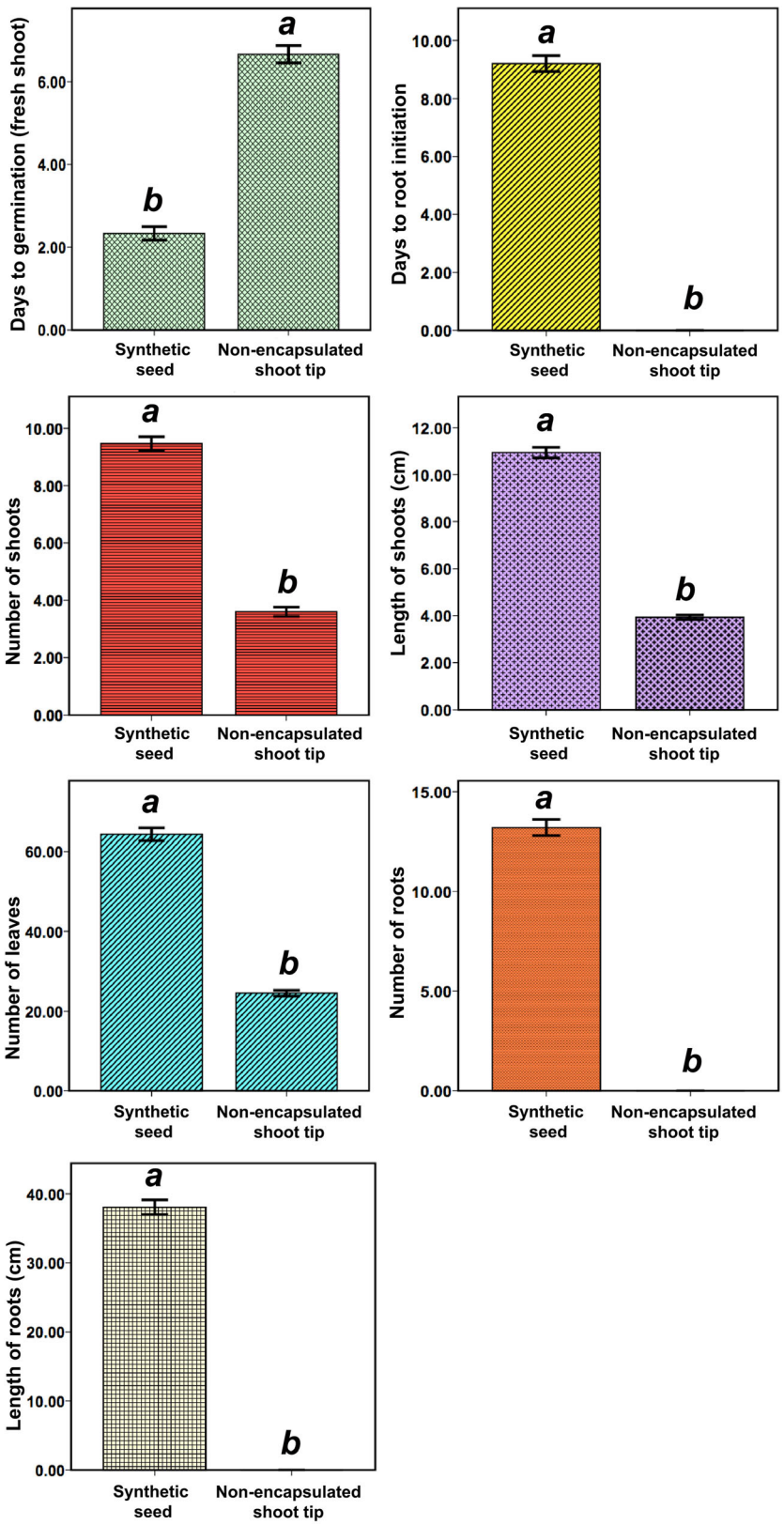

Fig. 3 Shoot-root initiation as well as various growth and developmental responses of alginate-encapsulated shoot tips (synthetic seeds) and non-encapulated shoot tips of *Stevia rebaudiana* Bert.** cultured on ½Murashige and Skoog liquid medium provided with filter paper M-bridge. Data represent mean ± standard error of 15 replications and 5 explants per replication. Data for each column followed by different alphabets are significantly different according to Tukey's test at $P = 0.05$. **Growth period of 30 days

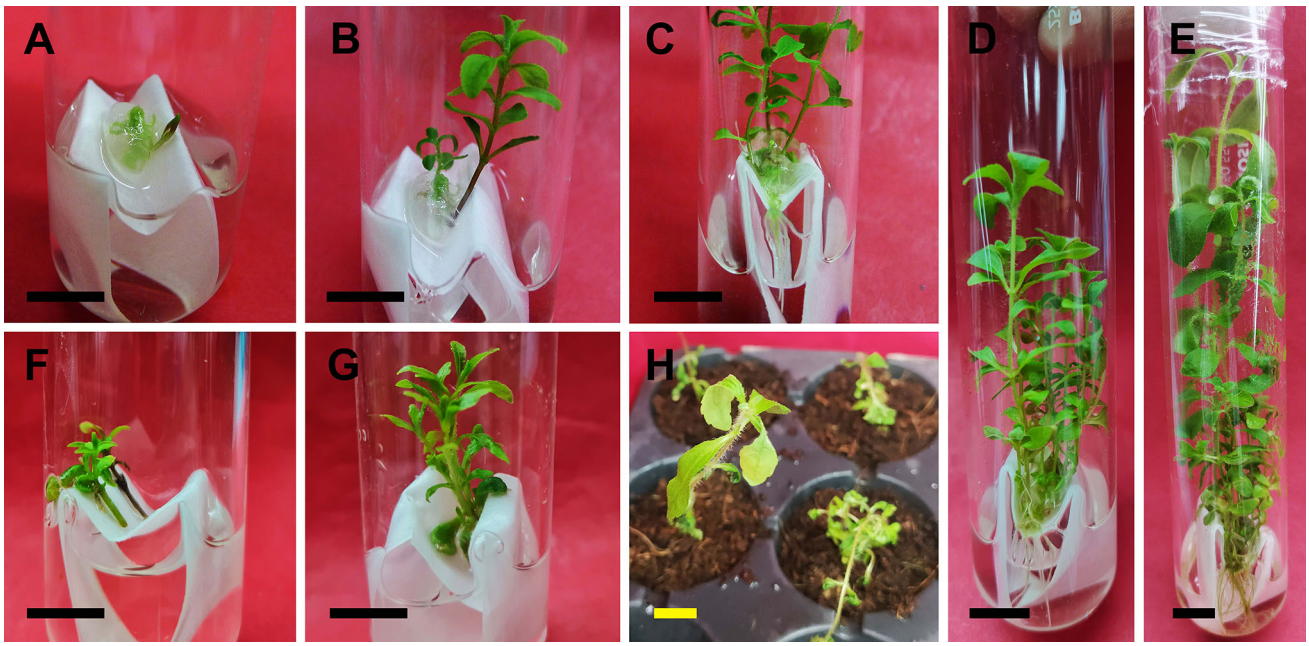

Fig. 4 Influence of alginate encapsulation (synthetic seeds) on growth and proliferation of shoot tips of *Stevia rebaudiana* Bert. **A** Initiation of germination (bud break) within ~ 2 days of inoculation in ½Murashige and Skoog liquid medium supported with M-bridge, **B** multiple shoot initiation after 7 days of inoculation, **C** root initiation and shoot elongation after ~ 9 days of inoculation, **D** mass multiplication, elongation and proliferation of shoots and foliage after 20 days of inoculation, **E** complete plantlet development at 30 days of inoculation, **F** delayed fresh shoot initiation from non-encapsulated shoot tips after 7 days of inoculation, **G** fewer shoots with reduced growth from non-encapsulated shoot tips after 30 days of inoculation, **H** acclimatization of synthetic seed-derived complete plantlets in cocopeat. (*Bar* = 10 mm)

growth and development of the plantlets in addition to minimizing the chance of soil-borne root and fungal diseases. There were merely few reports available on acclimatization of in vitro synthetic seed-derived plantlets of stevia. Lata et al. (2013) reported acclimatization of well-developed plantlets (with 87% survival rate) regenerated from alginate-encapsulated nodal segments of stevia. Recently, Shaafi et al. (2021) also reported acclimatization of shoots regenerated from encapsulated lateral buds of stevia in three different substrata like cocopeat, perlite and potting soil.

Assessment of Clonal Fidelity of Regenerants

A total of 10 ISSR primers that showed positive reaction were used to detect the genetic integrity of in vitro regenerants produced from encapsulated shoot tips (synthetic seeds) among themselves as well as with the mother plant. It was found that all the regenerants produced from encapsulated and non-encapsulated shoot tips obtained from mother plant produced monomorphic bands. Amplification with all 10 ISSR primers produced total 57 bands per sample with 5.7 as the average number of scorable bands per primer with band size ranging from 220 bp (in case of ISST-4, ISST-10) to 2300 bp (ISST-2) (Table 1). ISST-4 primer amplified with maximum number of 10 bands per sample. A total of 342 scorable monomorphic bands were produced from encapsulated shoot tips and mother plant that displayed 100% genetic integrity without deviation in the banding pattern (Fig. 5). List of all the 10 ISSR primers along with their sequences, Tm, number of scorable bands per primer per sample, total number of bands and their molecular sizes are given in Table 1. Gantait et al. (2017a) reported genetic fidelity (based on 97 monomorphic bands using 10 ISSR primers) of in vitro regenerants produced from the synthetic seeds of *R. serpentina*. Likewise, the sole report on stevia by Lata et al. (2016) affirmed the genetic integrity of germinated artificial seeds. However, it was less comprehensive, with a fewer number of bands (only 41) using 8 ISSR primers. There are other reports on molecular marker-based (chiefly random amplified polymorphic DNA; RAPD and ISSR) genetic fidelity assessment of synthetic seed-regenerated plantlets of several medicinal plant species such as on *Hedychium coronarium* using 7 RAPD and 8 ISSR primers (Behera et al. 2020), on *Bacopa monnieri* using ISSR primers (Pramanik et al. 2021), and most recently, on *R. serpentina* using 10 RAPD primers (Gantait et al. 2022).

Table 1 List of inter-simple sequence repeats (ISSR) primers used for assessment of genetic fidelity of regenerants obtained from synthetic seeds of *Stevia rebaudiana* Bert.

Primer(s)	Sequence (5′–3′)	T_m (°C)	Number of scorable bands /primer /sample	Total number of bands	Size range (bp)
ISST-1	$(CA)_8GT$	54	5	30	320–950
ISST-2	$(CA)_8AG$	54	4	24	750–2300
ISST-3	$(CA)_8AC$	54	6	36	400–1200
ISST-4	$(AC)_8$ T	50	10	60	220–1100
ISST-5	$(GGA)_8$	52	6	36	300–750
ISST-6	$(GTC)_5$	53	7	42	380–1250
ISST-7	$(CA)_6GT$	42	5	30	280–1100
ISST-8	$(CA)_6AG$	42	6	36	320–1300
ISST-9	$(CAC)_3GC$	45	4	24	350–1400
ISST-10	$CGC(GATA)_4$	45	4	24	220–450
Total			57	342	220–2370

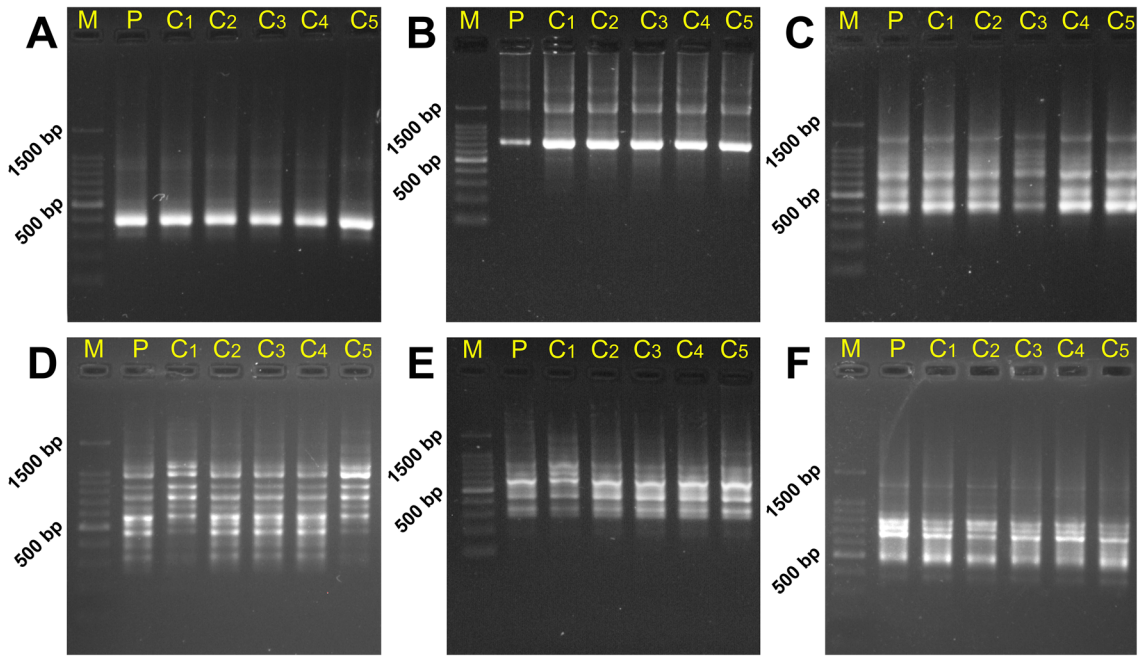

Fig. 5 Representative photographs of ISSR profiles of mother plant (P) and regenerants from synthetic seeds (C_1–C_5) of *Stevia rebaudiana* Bert. exhibiting monomorphic banding pattern with 6 ISSR primers, Lane 1: 100 bp ladder, Lane 2: DNA banding pattern of mother plant, Lane 3–7: DNA banding pattern of regenerants from synthetic seeds. **A** ISST-1 [$(CA)_8GT$], **B** ISST-2 [$(CA)_8AG$], **C** ISST-3 [$(CA)_8AC$], **D** ISST-4 [$(AC)_8T$], **E** ISST-5 [$(GGA)_8$], **F** ISST-6 [$(GTC)_5$]

Conclusion

In the present study, a simple protocol for multiple shoot-root regeneration of stevia is developed with a minimal concentration (0.5 mg/l) of BA. Most significantly, for the first time in stevia, we report an efficient, reproducible and a systematic procedure for enhancing regeneration of synthetic seeds and their subsequent growth and proliferation (required for germplasm exchange) in MSL basal media provided with M-bridge filter paper. The above-mentioned protocol eventually provides proper availability of nutrients to the synthetic seeds and helps in enhancement in regeneration as well as mass propagation of stevia. In addition, successful acclimatization and molecular marker-based genetic fidelity of synthetic seed-derived plantlets affirms the utility of such protocol. Our findings suggest that synthetic seed technology not only serves as a technique for germplasm storage and exchange

but can also enhance in vitro mass production of elite genotypes.

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Author Contributions TS was involved in methodology, data curation, formal analysis and original manuscript draft. **SL** was involved in methodology and data curation. **SNK** was involved in manuscript review and editing. **SS** was involved in manuscript review and editing. **SB** was involved in supervision, manuscript review and editing. **SG** was involved in conceptualization, funding acquisition, methodology, data curation, formal analysis, supervision, original manuscript draft, review and editing. All the authors read and approved the final version of the manuscript prior to its submission.

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Availability of Data and Materials All data generated or analysed during this study are included in this published article.

Declarations

Conflict of interests The authors declare that they have no competing interests.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

Aziz, R.A., and S.K. Al-Taweel. 2019. Effect of plant growth retardants on stevia (*Stevia rebaudiana* Bertoni) acclimatization produced in vitro. *Plant Archives* 19: 1275–1284.

Behera, S., K.K. Rout, P.C. Panda, and S.K. Naik. 2020. Production of non-embryogenic synthetic seeds for propagation and germplasm transfer of *Hedychium coronarium* J. Koenig. *Journal of Applied Research on Medicinal and Aromatic Plants* 19: 100.

Catana, C., and P. Boboc. 2021. In vitro liquid culture of *Stevia rebaudiana* Bert. for mass propagation. *Agro Life Scientific Journal* 10: 39–46.

Coelho, N., M.E. Gonzalez-Benito, and A. Romano. 2014. Cryopreservation of shoot tips from the endangered endemic species *Tuberaria major*. *Acta Physiologiae Plantarum* 36: 3333–3336.

Das, A., S. Gantait, and N. Mandal. 2011. Micropropagation of an elite medicinal plant: *Stevia rebaudiana* Bert. *International Journal of Agricultural Research* 6: 40–48.

Das, K., R. Dang, S. Khanam, B.G. Shivananda, and P.E. Rajasekharan. 2005. In vitro methods for production of stevioside from *Stevia rebaudiana*. *Indian Journal of Natural Products* 21: 14–15.

Faisal, M., A.A. Alatar, N. Ahmed, M. Anis, and A.K. Hegazy. 2012. Assessment of genetic fidelity in *Rauvolfia serpentina* plantlets grown from synthetic (encapsulated) seeds following in vitro storage at 4°C. *Molecules* 17: 5050–5061.

Gantait, S., A. Das, and J. Banerjee. 2018. Geographical distribution, botanical description and self-incompatibility mechanism of genus *Stevia*. *Sugar Tech* 20: 1–10.

Gantait, S., A. Das, and N. Mandal. 2015. Stevia: A comprehensive review on ethnopharmacological properties and in vitro regeneration. *Sugar Tech* 17: 95–106.

Gantait, S., S. Kundu, L. Yeasmin, and M.N. Ali. 2017a. Impact of differential levels of sodium alginate, calcium chloride and basal media on germination frequency of genetically true artificial seeds of *Rauvolfia serpentina* (L.) Benth. Ex Kurz. *Journal of Applied Research on Medicinal and Aromatic Plants* 4: 75–81.

Gantait, S., E. Mukherjee, P. Bandyopadhyay, and S. Bhattacharyya. 2022. M-brigde-and elicitor-assisted enhanced post-storage germination of *Rauvolfia serpentina* synthetic seeds, their genetic fidelity assessment and reserpine estimation. *Industrial Crops and Products* 180: 114732.

Gantait, S., and U.R. Sinniah. 2013. Storability, post-storage conversion and genetic stability assessment of alginate-encapsulated shoot tips of monopodial orchid hybrid *Aranda* Wan Chark Kuan 'Blue' × *Vanda coerulea* Grifft. Ex. Lindl. *Plant Biotechnology Reports* 7: 257–266.

Gantait, S., J. Vijayan, and A. Majee. 2017b. Artificial seed production of *Tylophora indica* (Burm. Fil.) Merrill for interim storing and swapping of germplasm. *Horticultural Plant Journal* 3: 41–46.

Hassanen, S.A., and R.M.A. Khalil. 2013. Biotechnological studies for improving of Stevia (*Stevia rebaudiana* Bertoni) in vitro plantlets. *Middle-East Journal of Scientific Research* 14: 93–106.

Karakas, F.P. 2020. Efficient plant regeneration and callus induction from nodal and hypocotyl explants of goji berry (*Lycium barbarum* L.) and comparison of phenolic profiles in calli formed under different combinations of plant growth regulators. *Plant Physiology and Biochemistry* 146: 384–391.

Kikowska, M., and B. Thiem. 2011. Alginate-encapsulated shoot tips and nodal segments in micropropagation of medicinal plants. A review. *Herba Polonica* 57: 45–57.

Kundu, S., U. Salma, M. Ali, and N. Mandal. 2018. Conservation, ex vitro direct regeneration, and genetic uniformity assessment of alginate-encapsulated nodal cuttings of *Sphagneticola calendulacea* (L.) Pruski. *Acta Physiologiae Plantarum* 40: 1–10.

Lata, H., S. Chandra, N. Techen, Y.H. Wang, M.A. ElSohly, and I.A. Khan. 2016. Genetic fidelity of *Stevia rebaudiana* Bertoni plants grown from synthetic seeds following in vitro storage. *Planta Medica* 82: PB23.

Lata, H., S. Chandra, Y.H. Wang, M.A. ElSohly, and I.A. Khan. 2013. *In vitro* germplasm conservation of elite *Stevia rebaudiana* Bertoni. *II International Symposium on Plant Cryopreservation* 1039: 303–308.

Murashige, T., and F. Skoog. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum* 15: 473–495.

Nower, A.A. 2014. In vitro propagation and synthetic seeds production: An efficient method for *Stevia rebaudiana* Bertoni. *Sugar Tech* 16: 100–108.

Pramanik, B., S. Sarkar, S. Bhattacharyya, and S. Gantait. 2021. meta-Topolin-induced enhanced biomass production via direct and indirect regeneration, synthetic seed production, and genetic fidelity assessment of *Bacopa monnieri* (L.) Pennell, a memory-booster plant. *Acta Physiologiae Plantarum* 43: 107.

Rafiq, M., M.U. Dahot, S.M. Mangrio, H.A. Naqvi, and I.A. Qarshi. 2007. *In vitro* clonal propagation and biochemical analysis of field established *Stevia rebaudiana* Bertoni. *Pakistan Journal of Botany* 39: 2467–2474.

Shaafi, B., S.S. Mosavi, M.R. Abdollahi, and H. Sarikhani. 2021. The optimized protocols for production, adaptation and keeping of the produced artificial seeds from encapsulated lateral buds in

Stevia rebaudiana (Bertoni). *Agrotechniques in Industrial Crops* 1: 24–35.

Siddique, I., and M. Anis. 2009. Morphogenic response of the alginate encapsulated nodal segments and antioxidative enzymes analysis during acclimatization of *Ocimum basilicum* L. *Journal of Crop Science and Biotechnology* 12: 233–238.

Singh, A.K., R. Varshney, M. Sharma, S.S. Agarwal, and K.C. Bansal. 2006. Regeneration of plants from alginate-encapsulated shoot tips of *Withania somnifera* (L.) Dunal, a medicinally important plant species. *Journal Plant Physiology* 163: 220–223.

Sivaram, L., and U. Mukundan. 2003. In vitro culture studies on (*Stevia rebaudiana*). *In Vitro Cellular Developmental and Biology-Plant* 39: 520–523.

Subrahmanyeswari, T., S. Gantait, S. Sarkar, and S. Bhattacharyya. 2022. Accelerated mono-phasic in vitro mass production of banana propagules and their morpho-cyto-genetic stability assessment. *South African Journal of Botany* 146: 794–806.

Tiwari, S., R. Arnold, A. Saxena, R.M. Mishra, A. Singh Tiwari, A. Rajak, and P. Singh. 2013. Studies on rapid micropropagation of *Stevia rebaudiana* Bertoni: A natural sweetener. *International Journal of Pharmacy and Life Sciences* 4: 2667–2671.