



Micropropagation and genetic fidelity of *Rheum ribes* L.: a threatened and endemic medicinal herb from the Turkish Eastern Anatolia region

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Abstract

Rheum ribes L., an endemic medicinal herb of Eastern Anatolia, faces serious conservation concerns due to habitat degradation, seed dormancy, and overharvesting. To overcome these challenges, a robust and reproducible in vitro regeneration system was developed using embryo axis, hypocotyl, and cotyledon explants via direct and indirect organogenesis as well as somatic embryogenesis. The highest germination rate (88.8%) and shortest germination time were obtained using ½ MS medium supplemented with 1.75 mg/L GA₃ and 1.0 mg/L IBA in mature embryos. The micropropagation experiments were conducted in two stages. In the first stage, embryo axis, cotyledon, and hypocotyl explants were cultured using 36 different methods that included various culture conditions and types of plant growth regulators at differing concentrations. In the second stage, calli from these explants were cultured in the most effective medium (method 30) for callus production, and the explants were subsequently cultured in eight different regeneration media based on MS medium supplemented with varying concentrations of plant growth regulators. Callus induction reached 100% under dark conditions in MS medium containing 0.5 mg/L BAP, 0.5 mg/L KIN, and 1.0 mg/L 2,4-D. Among 36 regeneration protocols, method 13 (3.0 mg/L BAP, 0.2 mg/L NAA, 1.0 mg/L GA₃) yielded the highest plant regeneration (1.15 plantlets/explant) in embryo axis and hypocotyl explants. In the second stage, the highest indirect somatic embryogenesis (ISER) rate (55.6%) was observed in cotyledon-derived callus (C-callus). In contrast, the highest indirect organogenesis (IOR) (28.1%) was found in embryo axis-derived callus (E-callus). The maximum number of plantlets per explant (PPEN: 1.08) was achieved in MS medium containing 2.0 mg/L BAP, 2.0 mg/L KIN, and 1.0 mg/L GA₃. Genetic fidelity of 15 regeneration systems was assessed using 20 highly polymorphic SCoT primers. Similarity coefficients between regenerated plants and the mother plant ranged from 0.904 to 0.991, with the highest genetic stability observed in plantlets derived via direct somatic embryogenesis. This optimized regeneration protocol is a powerful tool for the large-scale clonal propagation and ex-situ conservation of *R. ribes*, enabling both genetic preservation and sustainable biotechnological utilization of this pharmacologically valuable species.

Key message

Our research developed an effective in vitro regeneration system for the endemic and threatened medicinal plant *Rheum ribes* in Türkiye, enabling large-scale propagation while preserving its genetic integrity.

Keywords Plant regeneration · Embryo axis · Hypocotyl · Cotyledon · Organogenesis · Somaticembryogenesis · SCoT

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Introduction

Rheum ribes L. is an endemic medicinal plant belonging to the Polygonaceae family. It grows in the Eastern and South-eastern Anatolia Regions at altitudes of 1000–4000 m in April, May, and June (Rashid et al. 2014; Andiç et al. 2009). *Rheum ribes* is a rich source of minerals such as calcium, aluminum, phosphorus, iron, potassium, magnesium, selenium, zinc and copper, vitamins A, B, C, and E, and has

chemical components including chrysophanol, physcion, rutin, emodin, and aloe-emodin (Naemi et al. 2014; Malik et al. 2010; Sayyah et al. 2009; Munzuroğlu et al. 2000; Baytop 1997). *R. ribes* is also rich in tannin (Zeidali et al. 2021; Baytop 1999). Moreover, *Rheum* is rich in flavonoids, anthraquinones, anthocyanins, dianthrone, anti-glycosides, and essential oils (Ghasemi et al. 2024; Pandith and Khan 2022; Rokaya et al. 2012). Rhubarb species have a wide range of uses in traditional medicine (Roghani-Shahraki et al. 2021; Khiveh et al. 2017). The species of this plant are known among the public as antidiabetic, antibacterial, antispasmodic, anti-inflammatory, anti-Alzheimer, antioxidant, antitumor, and anticancer (Nehme et al. 2025; Yuan et al. 2020; Keser et al. 2019; Erbay and Sarı 2018; Rajkumar et al. 2010; Choi et al. 2005; Tosun and Kızılay 2003). In addition to the environmental stress these plants face during growth, excessive harvesting by people further threatens their survival. Gathering medicinal plants from the wild as a source of raw materials not only damages their natural habitats but also contributes to the loss of valuable genetic diversity (Halder and Jha 2023; Canter et al. 2005). *Rheum ribes* exhibit limited natural propagation due to several biological and ecological constraints. Seed germination rates are often low, primarily due to seed dormancy and environmental factors such as temperature and moisture availability (Akin et al. 2019; Nabaei et al. 2011). Moreover, the plant's reproductive success is hindered by overharvesting of immature plants before seed set, as well as habitat degradation, which reduces the availability of suitable germination sites. These limitations emphasize the need for developing efficient in vitro propagation systems, as described in our study (Coelho et al. 2020; Arvas 2017). Using these micropropagation techniques, many successful studies have been done previously for *R. palmatum* (Cui et al. 2008), *R. ribes* (Farzami Seppehr and Ghorbanli 2005), *R. rhabarbarum* (Clapa et al. 2020; Rayirath et al. 2011; Lepse 2007), *R. coreanum* (Mun and Mun 2016), *R. webbianum* (Rashid et al. 2014), *R. moorcroftianum* (Maithani 2015), *R. emodi* (Singh and Chaturvedi 2022; Tabin et al. 2018) and *R. spiciforme* (Khan et al. 2022). The success of plant micropropagation depends mainly on many factors such as the content of the nutrient medium, sterilization, explant and genotype. The age, species and location of the explant are also very important for both somatic embryogenesis and organogenesis (Andrade et al. 2024; Desai et al. 2022; Shahzad et al. 2017; Aydın et al. 2011; Kumar et al. 2002).

Somatic embryogenesis is considered one of the most used regeneration pathways of the tissue culture system (Aronen et al. 2025; Sánchez-Romero 2021; Tomiczak et al. 2019; Aydın et al. 2013; Chand and Sahrawat 2002).

Previous studies on the *Reham* genus micropropagation used various combinations of cytokinin and auxin. Khan

et al. (2022) used the combination of NAA and BAP was used for shoot formation, while the combination of 2,4-D and BAP for callus formation. On the other hand, only BAP, KIN, and meta-Topolin (mT) cytokinins were used for shoot formation, and successful results were reported (Kozak and Saáata 2011; Clapa et al. 2020). In some studies, callus has been induced using only NAA and 2,4-D (Mun and Mun 2016; Maithani 2015).

The acclimatization phase, which is the last phase of every micropropagation protocol, is the process of transferring regenerated plants to the soil in natural environmental conditions (Grzelak et al. 2024). The main goal in micropropagation studies is to obtain plants with the same genetic makeup as the parent plant. However, undesirable differences called somaclonal variations may occur in the regeneration processes for micropropagation studies. These variations may be caused by factors like plant growth regulators, genotype, and culture period, and they can be detected by molecular, morphological, and biochemical methods, but molecular techniques can determine genetic instability at earlier stages (Duta-Cornescu et al. 2023; Govindaraj 2015). For this purpose, DNA-based molecular markers, such as RFLP, AFLP, RAPD, SSR, ISSR and SCoT (Start Codon Targeted) are used to assess genetic stability (Elshibli and Korpelainen 2007; Khan et al. 2012; Kudikala et al. 2020; Tikendra et al. 2021). SCoT marker is a simple and reproducible technique that targets regions around the ATG start codon in the plant genome (Collard and Mackill 2009). This technique has been successfully used in plants such as peanut (Luo et al. 2010), wheat (Etminan et al. 2016), sugar cane (Sathish et al. 2018), barley (Güngör et al. 2022), and Heart-wing Sorrel (Singh et al. 2025).

Studies on micropropagation in *Rheum ribes* have been limited, particularly in terms of organogenesis and somatic embryogenesis. In the current study, an effective micropropagation system was developed using these methods, and then somaclonal variations in the regenerated plants were evaluated using the SCoT marker technique.

Materials and methods

Plant material and sterilization

Seeds of *Rheum ribes* L. were collected from wild populations in Erzurum, located at an altitude of 2,450 m (39°26'26.6"N, 42°09'55.7"E). The seeds were stored at 4°C until used in tissue culture. Before sterilization, the seed wings were manually removed without damaging the embryo, as the seeds have a large, winged structure that can prevent chemicals from penetrating the seed (Tuncer and Günsan 2017). After washing the seeds for ten minutes

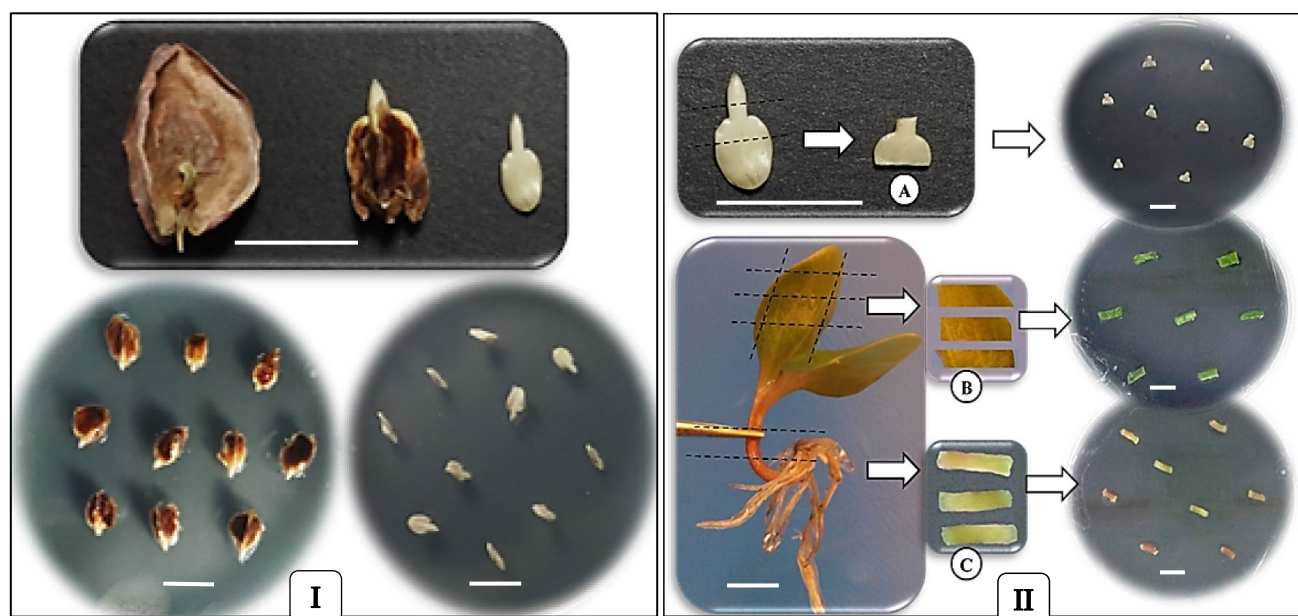


Fig. 1 **I** Seed and embryo explants used in germination; **II** Explants used in the first stage (A) Embryo axis, (B) Cotyledon, (C) Hypocotyl, Scale bars = 1 cm

Table 1 Germination rate (%) and average germination time (days) of seed and embryo explants in different media

Medium	GA3 (mg/l)	IBA (mg/l)	Germination rate (%)			Average germination time (Day)		
			Seed	Embryo	Average	Seed	Embryo	Average
MS	0.0	0.0	27.50 ^c	47.50 ^c	37.50 ^d	13.75 ^a	8.75 ^a	11.25 ^a
MS	0.0	1.0	40.00 ^d	60.00 ^d	50.00 ^c	12.25 ^b	8.00 ^{abc}	10.13 ^b
MS	1.75	0.0	52.50 ^c	52.50 ^{de}	52.50 ^c	11.00 ^c	7.25 ^{cde}	9.13 ^c
MS	1.75	1.0	67.50 ^{ab}	70.00 ^c	68.75 ^b	10.25 ^{cd}	7.00 ^{cde}	8.63 ^{cd}
½ MS	0.0	0.0	57.50	52.50 ^{de}	55.00 ^c	9.75 ^d	8.50 ^{ab}	9.13 ^c
½ MS	0.0	1.0	60.00 ^{bc}	80.00 ^b	70.00 ^b	9.25 ^d	7.50 ^{bcd}	8.38 ^d
½ MS	1.75	0.0	52.50 ^c	92.50 ^a	72.50 ^b	7.75 ^e	6.50 ^{de}	7.13 ^e
½ MS	1.75	1.0	77.50 ^a	100.00 ^a	88.75 ^a	7.25 ^e	6.25 ^e	6.75 ^e
Average			54.38 ^B	689.3 ^A		10.16 ^A	7.47 ^B	
F value (Explant -E)			—	—	230.40 ^{**}	—	—	528.29 ^{**}
F value (Medium-M)			51.33 ^{**}	124.43 ^{**}	133.03 ^{**}	86.84 ^{**}	14.84 ^{**}	79.35 ^{**}
F value (E × M)			—	—	15.63 ^{**}	—	—	22.33 ^{**}
CV (%)			7.96	5.09		4.61	6.26	

Differences between averages shown with the same lower-case letter in the same column and the same upper-case letter in the same row are insignificant ($p > 0.05$). **: Significant at 0.01 level.

under tap water, they were sterilized with 70% ethyl alcohol for 1 min, then rinsed 3 times with sterile water and mixed in a 5% sodium hypochlorite solution with Tween-20 for 10 min, then rinsed with sterile water. After that, the seeds were stored in pure distilled water at +4 °C for 24 h.

Murashige and Skoog's (1962) medium (MS) was used as the basal nutrient medium in all stages of the research. MS salt medium (Sigma M5524 was sterilized by autoclaving at 121 °C and 15 psi (105 kPa) pressure for 15 min. 0.22 µm porous cellulose nitrate filters were used to sterilize components that can be degraded at high temperatures, such

as MS vitamins (Sigma M3900) and plant growth regulators. The pH was adjusted to 5.8 prior to autoclave.

Seed and embryo germination

The mature embryos of the sterilized seeds were removed in a sterile cabinet with forceps and scalpels without damaging the embryos. Then, whole seed and embryo explants (Fig. 1) were cultured in 8 different media containing ½ Murashige and Skoog (1962) medium (MS), 30 g/l sucrose, 1.95 mg/l MES hydrate, 7 g/l agar, GA3 and IBA for germination (Table 1) at 25 ± 1 °C and 16:8 h photoperiod for 14

days (Tuncer and Günsan 2017; Khan et al. 2022; Singh and Chaturvedi 2022; Tuncer 2021). Germination rate (%) and Average Germination Time (days) were determined using methods described by Orhan et al. (2020).

First stage of micropropagation

In the first stage, embryo axis explants obtained from mature seeds, as well as hypocotyl and cotyledon explants taken from 7-day-old seedlings germinated under in vitro conditions from the embryo explants, were cultured using 36 different methods. These methods varied in culture conditions and MS basal medium containing various types and concentrations of plant growth regulators (PGRs) (Table 2). The method used in this study was adapted and modified from protocols employed by various researchers working on rhubarb (*Rheum ribes*) and other species in the *Rheum* genus (Khan et al. 2022; Singh and Chaturvedi 2022; Wojtania and Mieszczakowska-Frać 2021; Tabin et al. 2018).

In these methods, the PGRs were used in combination with MS medium, 30 g/L sucrose, 7 g/L agar, 1.95 g/L MES hydrate, and 0.7 mg/L Polyvinylpyrrolidone (PVP), as listed in Table 2. In methods 1–29, the explants were cultured in the dark at 25 ± 1 °C for 14 days. After this period, they were subcultured into the same medium and incubated under a 16-h photoperiod ($32 \mu\text{mol}/\text{m}^2/\text{s}$) at 25 ± 1 °C for 21 days.

In methods 30–36, explants were cultured in the dark at 25 ± 1 °C for 14 days, then subcultured into the same medium on the 15 th day and maintained in the dark for a total of 35 days. Each Petri dish was considered a replicate, with 10 explants per dish. This stage of the experiment was conducted with four replicates according to a completely randomized experimental design.

Second stage of micropropagation

In the first stage, calli obtained from embryo axis, hypocotyl, and cotyledon explants on day 14 in method 30 [embryo axis-derived callus (E-callus), hypocotyl-derived callus (H-callus) and cotyledon-derived callus (C-callus)] were used as explants at the second stage. In these media, PGRs used in addition to MS medium, 30 g/l sucrose, 7 g/l agar + 1.95 g/l MES hydrate, and 0.7 mg/l PVP are given in Table 2. Explants were cultured in the dark at 25 ± 1 °C for 14 days. At the end of the 14 th day, they were sub-cultured in the same medium at 25 ± 1 °C with a 16-h photoperiod ($32 \mu\text{mol}/\text{m}^2/\text{s}$) for 21 days.

Rooting of shoots and acclimation

Shoots that reached approximately 2–2.5 cm in length were rooted in magenta boxes in $\frac{1}{2}$ MS + 1 mg/l IBA + 30 g/l sucrose + 1.95 g/l MES hydrate + 7 g/l agar (Farzami Sepehr

Table 2 MS basal medium supplemented with various types and concentrations of plant growth regulators (PGRs) in micropropagation methods and medium

First Stage/embryo axis, cotyledon, and hypocotyl explants			
Method	PGR combination (mg/l)	Method	PGR combination (mg/l)
1	1.0 NAA	19	4.0 KIN + 2.0 NAA
2	2.0 NAA	20	4.0 KIN + 0.2 NAA + 1.0 GA3
3	1.0 BAP	21	5.0 KIN + 0.2 NAA + 1.0 GA3
4	2.0 BAP	22	6.0 KIN + 0.2 NAA + 1.0 GA3
5	3.0 BAP	23	4.0 BAP + 1.0 2,4-D
6	1.0 KIN	24	5.0 BAP + 2.0 2,4-D
7	2.0 KIN	25	2.0 BAP + 1.0 IBA
8	3.0 KIN	26	3.0 BAP + 2.0 IBA
9	2.0 BAP + 1.0 KIN + 0.5 TDZ + 1.0 GA3	27	3.0 BAP + 1.0 IBA
10	2.0 BAP + 0.5 NAA	28	3.0 BAP + 0.5 IBA
11	3.0 BAP + 2.0 NAA	29	2.0 KIN + 1.0 IBA
12	3.0 BAP + 1.0 NAA	30	0.5 BAP + 0.5 KIN + 1.0 2,4-D
13	3.0 BAP + 0.2 NAA + 1.0 GA3	31	1.0 BAP + 1.0 2,4-D
14	4.0 BAP + 0.2 NAA + 1.0 GA3	32	1.0 BAP + 2.0 2,4-D
15	5.0 BAP + 0.2 NAA + 1.0 GA3	33	2.0 BAP + 1.0 2,4-D
16	6.0 BAP + 0.2 NAA + 1.0 GA3	34	2.0 BAP + 2.0 2,4-D
17	3.0 KIN + 1.0 NAA	35	2.0 KIN + 2.0 2,4-D
18	3.0 KIN + 0.2 NAA + 1.0 GA3	36	1.0 BAP + 1.0 KIN + 1.0 2,4-D
Second Stage/E-callus, H-callus ve C-callus explants*			
Medium	PGR Combination (mg/l)	Medium	PGR Combination (mg/l)
1	2.0 BAP + 0.5 TDZ	5	2.0 BAP + 2.0 KIN + 0.2 NAA + 1.0 GA3
2	2.0 BAP + 0.2 NAA + 1.0 GA3	6	2.0 BAP + 2.0 KIN + 1.0 GA3
3	2.0 BAP + 0.1 IAA + 1.0 GA3	7	3.0 BAP + 0.1 KIN + 0.1 IAA
4	2.0 BAP + 0.1 NAA + 0.1 IAA	8	2.0 BAP + 1.0 IBA

*E-callus: embryo axis-derived callus, H-callus: hypocotyl-derived callus, C-callus: cotyledon-derived callus

and Ghorbanli 2005). The obtained plants were taken from the *in vitro* culture medium and adapted to the external environment. To remove nutrient medium residues from the root parts of the plants, they were washed under tap water without damaging the root part. The plants whose root parts were washed were planted in small pots containing 1:1 peat and soil, covered with a nylon bag, and watered with $\frac{1}{4}$ Hoagland solution (Hoagland and Arnon 1950) under the pot. The plants in the pots continued to grow at 25 ± 1 °C with a 16-h photoperiod. At the end of 1 week, the nylon bags were gradually punctured. At the end of 10 days, the young plants were acclimatized to the environmental conditions by removing them for 1–2 h a day in the first days, then for 4–5 h, and completely after 4 weeks. The acclimated plants were transferred into larger pots containing fresh, sterile garden soil to support further growth and subsequently maintained under greenhouse conditions.

Determination of somaclonal variation

In the study, the genetic stability of plants obtained from systems with the highest number of regenerated plants in terms of PPEN in the 1st and 2nd stages was determined by the SCoT molecular marker technique. A bulk DNA sampling strategy was applied in the study (Reyes-Valde et al. 2013). Leaf samples were collected from three randomly selected regenerated plantlets within each replicate of every regeneration system. As each system consisted of four replicates, 12 plantlets were sampled per regeneration system to form a bulk sample for analysis. Leaf samples frozen in liquid nitrogen were stored at -80 °C until DNA isolation. The DNA was extracted using the cetyltrimethylammonium bromide (CTAB) technique reported by Cota-Sánchez et al (2006). The quantity and purity of the extracted DNA were evaluated by Nanodrop spectrophotometry (Thermo Multiskan Go). DNA samples were kept at -20 °C until usage. SCoT primers were determined by literature research (Collard and Mackill 2009). Thirty SCoT primers were tested in the study (Xia et al. 2016) from which the twenty primers showing the highest quality amplification profile were selected (Table 7). SCoT-PCR process was carried out by a thermal cycler (Thermo Scientific 5020). The PCR reaction was in a final volume of 15 μ L containing 1 μ L (10 ng) of genomic DNA, 8 μ L of Taq DNA Master Mix, 1 μ L primer, and 5 μ L ddH₂O. The SCoT-PCR conditions were as follows: 5 min at 95 °C, 40 cycles of 1 min at 94 °C, 1 min at 54–63 °C (primer annealing), 1.5 min at 72 °C, ending with a final extension step of 10 min at 72 °C, the program was terminated at 4 °C for 10 min. The amplified PCR products (10 μ L) mixed with 6 $\times$ gel loading buffer (3 μ L) were loaded in an agarose gel (1.5% w/v) containing 2 μ L ethidium bromide and the electrophoresis was conducted in 1X

TBE (Tris–borate–EDTA) buffer at 70 V for 1.5 h. Then the DNA bands were visualized by UV transillumination.

Statistical analyses

The data of the parameters examined in the first stage of the study were subjected to analysis of variance (ANOVA) in a 3 (explant type) $\times$ 36 (method) factorial design, and the data of the parameters examined in the second stage were subjected to analysis of variance (ANOVA) in a 3 (explant type) $\times$ 8 (medium) factorial design according to a completely random experimental plan. Differences between means were determined with the Tukey multiple comparison test at a significance level of 5%. Statistical analyses were performed using the Minitab 19 computer program. A binary matrix was created in the SCoT amplification bands with the help of the Total Lab TL120 computer program as present (1) or absent (0). The data obtained were analyzed with the NTSYSpc 2.11 package program. The Jaccard similarity coefficient was calculated from the binary matrices using the SIMQUAL module. A dendrogram was created from the similarity coefficients using the UPGMA method (unweighted paired group method using arithmetic mean) using the SAHN module. Polymorphism information content (PIC), resolving power (R), and discrimination power (D) for each primer were determined by online iMEC software (Amiryousefi et al. 2018).

Results

First stage of micropropagation

In vitro germination

The research investigated the influence of different strengths of MS basal medium (MS and $\frac{1}{2}$ MS), along with varying concentrations of gibberellic acid (GA₃; 0 and 1.75 mg/L) and indole-3-butyric acid (IBA; 0.0 and 1.0 mg/L), on seed and embryo germination performance. The results demonstrated statistically significant differences ($p < 0.01$) in both germination rates and time across the tested treatments. Embryos exhibited a higher germination rate (69.4%) compared to seeds (54.4%). The best results were obtained using the $\frac{1}{2}$ MS medium supplemented with 1.75 mg/l GA₃ and 1.0 mg/l IBA, which yielded the highest overall germination rate of 88.8% (Fig. 2). Germination rates for embryos ranged widely, from 47.5% up to 100%, while seeds ranged from 27.5% to 77.5%. Additionally, embryos germinated faster, with an average germination time of 7.5 days, compared to 10.2 days for seeds. The shortest germination time (6.75 days) was also recorded in the optimal medium for

Fig. 2 In vitro germination of seed and embryo explants. **A** Seed with MS + 0.0 mg/l GA3 + 0.0 mg/l IBA. **B** Embryo with $\frac{1}{2}$ MS + 1.75 mg/l GA3 + 1.0 mg/l IBA. **C** Seed with MS + 0.0 mg/l GA3 + 1.0 mg/l IBA. **D** Embryo with $\frac{1}{2}$ MS + 1.75 mg/l GA3 + 0.0 mg/l IBA, Scale bars = 1 cm



both embryos and seeds (Table 1). These results suggest that using embryos in the optimal medium is effective for both high germination rates and reduced germination times.

Callus formation rate (CFR%)

The study investigated callus formation across different structures and colors, revealing that somatic embryogenesis and organogenesis primarily occurred in green and light green calli, while yellow, white, and watery calli appeared loose and soft, and red calli were non-viable (Fig. 3). Callus formation responses (CFR) were evaluated using 36 different methods across three explant types—embryo axis, hypocotyl, and cotyledon—revealing statistically significant interactions between explant type and method ($p < 0.01$) (Table 3). The average CFR was 73.3% for the cotyledon, 72.9% for the hypocotyl, and 67.2% for the embryo axis. Differences between the cotyledon and hypocotyl were statistically insignificant, but both were significantly higher than the embryo axis ($p < 0.01$). Method 30 (MS medium containing 0.5 mg/l BAP + 0.5 mg/l KIN + 1.0 mg/l 2,4-D in dark conditions) yielded the highest average CFR at 100%, while method 12 showed the lowest at 35%. The CFR varied

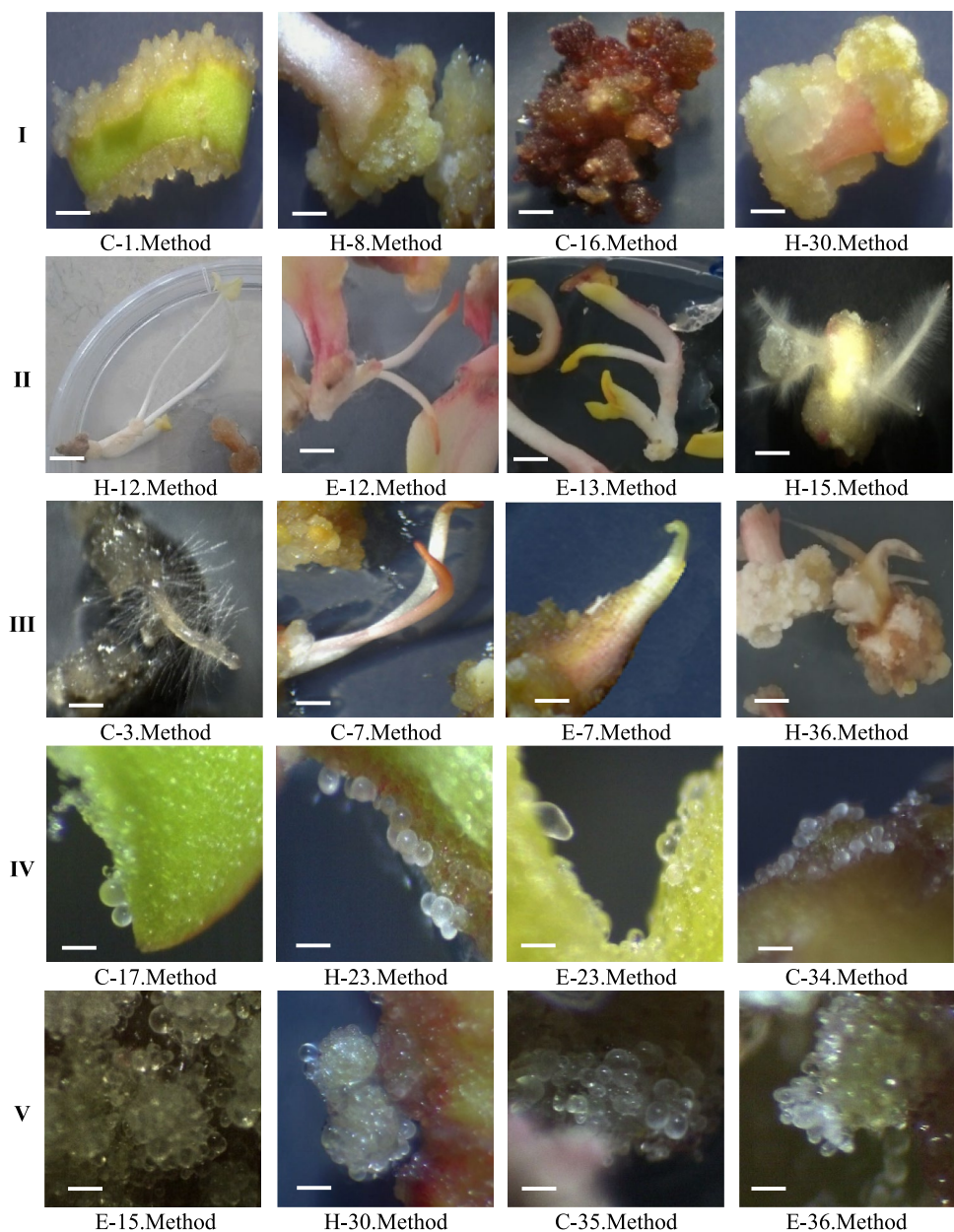
by explant type, ranging from 17.5% to 100% for embryo, 25.0% to 100% for hypocotyl, and 42.5% to 100% for cotyledon, with method 30 consistently achieving the highest rates across all explants (Table 3).

Organogenesis rate (%)

Direct organogenesis rate (DOR) (%) measures the capacity of explants to form shoots or roots directly without callus formation. The direct organogenesis rate (DOR) was calculated as follows: $\text{DOR (\%)} = (\text{Number of explants producing direct shoots or roots} / \text{Total number of explants}) \times 100$.

The study found a significant impact of explant type on DOR ($p < 0.01$) with the embryo axis achieving the highest rate at 12.6%, followed by hypocotyl (8.8%) and cotyledon (8.2%) (Fig. 3). Significant differences were also noted among the methods used. Direct organogenesis did not occur in 23 methods, while those that did showed DOR rates ranging from 10.0% (method 14) to 59.2% (method 13). The interaction between explant type and method was significant ($p < 0.01$). For methods where direct organogenesis was successful, DOR rates varied for each explant:

Fig. 3 I: Callus formation in different explants and methods (Explant-Method). **II:** Direct organogenesis. **III:** Indirect organogenesis. **IV:** Direct somatic embryogenesis. **V:** Indirect somatic embryogenesis. C: Cotyledon; H: Hypocotyl. E: Embryo axis. Explanations of method numbers are given in Table 2. Scale bars: I, II and II = 1 mm, IV and V = 0.5 mm



embryo axis from 10.0% to 97.5%, hypocotyl from 10.0% to 50.0%, and cotyledon from 10.0% to 47.5% (Table 3).

Indirect organogenesis rate (IOR %) measures the ability of explants to form shoots or roots from callus. The IOR was calculated as follows: $\text{IOR (\%)} = (\text{Number of explants producing indirect shoots or roots} / \text{Total number of explants}) \times 100$. A significant effect of explant type on IOR was found ($p < 0.01$), with cotyledon explants yielding a higher IOR of 7.4% compared to the other two explants, which had an IOR of 6.6%, indicating a significant difference ($p < 0.01$). Indirect organogenesis did not occur in 14 methods, while the IOR for successful methods ranged from 3.3% (methods 13, 19, and 33) to 26.7% (method 36) (Fig. 3). Specifically, 22 methods failed for the embryo axis, 21 for hypocotyl,

and 20 for cotyledon. For methods where indirect organogenesis was successful, IOR rates varied in all used explants between 10.0% and 30.0% (Table 3).

Somatic embryogenesis rate (%)

Direct somatic embryogenesis rate (DSER%) measures the ability of explants to form direct somatic embryos without callus formation. Direct somatic embryogenesis rate (DSER) was calculated as follows: $\text{DSER (\%)} = (\text{Number of explants producing direct somatic embryogenesis} / \text{Total number of explants}) \times 100$. A significant effect of explant type on DSER was observed ($p < 0.01$), with hypocotyl achieving the highest rate at 40.0%, followed closely by

Table 3 Callus formation rate (CFR %) and colors, direct and indirect organogenesis formation (DOR and IOR %), direct and indirect somatic embryogenesis formation (DSER and ISER %) in different explants and methods

Method	Embryo axis						Hypocotyl						Cotyledon						Average																	
	CFR			C-Color			DOR			IOR			DSER			ISER			CFR			C-Color			DOR			IOR			DSER			ISER		
	%			%			%			%			%			%			%			%			%			%			%					
1	45.0 ^{ijk}	LY	0.0 ^b	LY	0.0 ^b	LY	0.0 ^e	0.0 ^e	0.0 ^j	10.0 ^e	0.0 ^e	0.0 ^e	55.0 ^{lm}	LY	0.0 ^f	LY	0.0 ^f	0.0 ^e	0.0 ^e	0.0 ⁿ	20.0 ^{ad}	52.5 ^{kl}	0.0 ^b	0.0 ^b	0.0 ^b	0.0 ^b	0.0 ^b	0.0 ^b	0.0 ^b	0.0 ^b	0.0 ^b	10.0 ^h				
2	35.0 ^k	LY	0.0 ^b	LY	0.0 ^b	LY	0.0 ^e	0.0 ^e	0.0 ^j	20.0 ^d	20.0 ^d	0.0 ^e	45.0 ^{ij}	Y	0.0 ^f	LY	0.0 ^f	30.0 ^a	30.0 ^a	0.0 ⁿ	0.0 ⁿ	20.0 ^{ad}	49.2 ^{lm}	0.0 ^b	10.0 ^f	10.0 ^f	0.0 ^b	10.0 ^f	0.0 ^b	8.3 ^p	16.7 ^{ef}	20.0 ^d				
3	32.5 ^k	LR	0.0 ^b	LY	0.0 ^b	LY	0.0 ^e	0.0 ^e	10.0 ^{hi}	20.0 ^d	20.0 ^d	0.0 ^e	42.5 ^{ij}	Y	0.0 ^f	LY	0.0 ^f	5.0 ^f	0.0 ^e	10.0 ^{mm}	0.0 ⁿ	40.8 ^{mm}	0.0 ^b	40.8 ^{mm}	0.0 ^b	0.0 ^b	0.0 ^b	0.0 ^b	0.0 ^b	8.3 ^p	16.7 ^{ef}	20.0 ^d				
4	65.0 ^{e-h}	Y	20.0 ^f	LY	20.0 ^f	LY	0.0 ^e	0.0 ^e	37.5 ^{efg}	20.0 ^d	20.0 ^d	0.0 ^e	55.0 ^{ghi}	LY	10.0 ^e	LY	10.0 ^e	0.0 ^e	0.0 ^e	52.5 ^{e-f}	30.0 ^b	59.2 ^{ijk}	13.3 ^f	0.0 ^b	10.0 ^f	10.0 ^f	0.0 ^b	10.0 ^f	0.0 ^b	45.0 ^{e-h}	23.3 ^c	6.7 ^{ij}				
5	52.5 ^{bij}	W	0.0 ^b	LY	10.0 ^d	LY	0.0 ^e	20.0 ^b	47.5 ^{ade}	10.0 ^e	10.0 ^e	0.0 ^e	67.5 ^{d-g}	G	0.0 ^f	LY	0.0 ^f	20.0 ^b	20.0 ^b	47.5 ^{de}	0.0 ^f	63.3 ^{bij}	0.0 ^b	63.3 ^{bij}	0.0 ^b	10.0 ^f	10.0 ^f	0.0 ^b	10.0 ^f	0.0 ^b	49.2 ^{e-f}	6.7 ^{ij}				
6	62.5 ^{gh}	B	0.0 ^b	LY	0.0 ^b	LY	0.0 ^e	0.0 ^e	20.0 ^{hi}	0.0 ^e	0.0 ^e	0.0 ^e	25.0 ^k	B	0.0 ^f	LY	0.0 ^f	0.0 ^e	0.0 ^e	20.0 ^k	17.5 ^{lm}	43.3 ^{mm}	0.0 ^b	43.3 ^{mm}	0.0 ^b	0.0 ^b	0.0 ^b	6.7 ^{ij}	19.2 ^c	6.7 ^{ij}	6.7 ^{ij}					
7	57.5 ^{gh}	W	0.0 ^b	LY	20.0 ^b	LY	0.0 ^e	20.0 ^b	52.5 ^{ad}	10.0 ^e	10.0 ^e	0.0 ^e	35.0 ^{jk}	LY	0.0 ^f	LY	0.0 ^f	10.0 ^d	30.0 ^a	45.0 ^{de}	10.0 ^e	48.3 ^{mm}	0.0 ^b	48.3 ^{mm}	0.0 ^b	20.0 ^b	20.0 ^b	0.0 ^b	48.3 ^{mm}	16.7 ^{ef}	16.7 ^{ef}	16.7 ^{ef}				
8	52.5 ^{gh}	W	0.0 ^b	LY	0.0 ^b	LY	0.0 ^e	0.0 ^e	37.5 ^{efg}	10.0 ^e	10.0 ^e	0.0 ^e	55.0 ^{ghi}	Y	0.0 ^f	LY	0.0 ^f	0.0 ^e	0.0 ^e	45.0 ^{de}	10.0 ^e	55.0 ^{gh}	0.0 ^b	55.0 ^{gh}	0.0 ^b	0.0 ^b	0.0 ^b	0.0 ^b	41.7 ^{gh}	3.3 ^k	3.3 ^k	3.3 ^k				
9	87.5 ^{abc}	Y	0.0 ^b	LY	0.0 ^b	LY	0.0 ^e	10.0 ^d	52.5 ^{ad}	20.0 ^d	20.0 ^d	0.0 ^e	85.0 ^{abc}	Y	0.0 ^f	LY	0.0 ^f	10.0 ^d	10.0 ^d	55.0 ^{de}	17.5 ^{lm}	83.3 ^{kl}	0.0 ^b	83.3 ^{kl}	0.0 ^b	10.0 ^f	10.0 ^f	0.0 ^b	10.0 ^f	55.0 ^{bed}	20.0 ^d	20.0 ^d	20.0 ^d			
10	65.0 ^{e-h}	LY	30.0 ^e	LY	30.0 ^e	LY	0.0 ^e	0.0 ^e	35.0 ^{fg}	0.0 ^f	0.0 ^f	0.0 ^e	85.0 ^{abc}	W	27.5 ^e	LY	10.0 ^e	0.0 ^e	0.0 ^e	47.5 ^{de}	20.0 ^e	77.5 ^{fg}	22.5 ^e	0.0 ^b	0.0 ^b	0.0 ^b	0.0 ^b	0.0 ^b	45.8 ^{e-h}	13.3 ^g	13.3 ^g	13.3 ^g				
11	57.5 ^{gh}	R	0.0 ^b	LY	0.0 ^b	LY	0.0 ^e	0.0 ^e	20.0 ^{hi}	0.0 ^f	0.0 ^f	0.0 ^e	62.5 ^{e-h}	LB	0.0 ^f	LY	0.0 ^f	0.0 ^e	0.0 ^e	20.0 ^k	37.5 ^{de}	60.8 ^{ijk}	0.0 ^b	60.8 ^{ijk}	0.0 ^b	0.0 ^b	0.0 ^b	0.0 ^b	19.2 ^c	19.2 ^c	19.2 ^c	19.2 ^c				
12	17.5 ^l	LG	97.5 ^a	LY	0.0 ^b	LY	0.0 ^e	0.0 ^e	0.0 ^j	0.0 ^f	0.0 ^f	0.0 ^e	45.0 ^{ij}	W	30.0 ^{bc}	LY	20.0 ^d	0.0 ^e	0.0 ^e	47.5 ^{de}	0.0 ^f	35.0 ^{lm}	49.2 ^b	0.0 ^b	0.0 ^b	0.0 ^b	0.0 ^b	0.0 ^b	35.8 ^{kl}	3.3 ^k	3.3 ^k	3.3 ^k				
13	92.5 ^{ab}	LR	80.0 ^b	LY	0.0 ^b	LY	0.0 ^e	0.0 ^e	20.0 ^{hi}	0.0 ^f	0.0 ^f	0.0 ^e	92.5 ^{ab}	LG	50.0 ^a	LY	0.0 ^f	0.0 ^e	0.0 ^e	65.0 ^{ab}	20.0 ^e	90.8 ^{bed}	59.2 ^a	0.0 ^b	0.0 ^b	0.0 ^b	0.0 ^b	0.0 ^b	27.5 ^{mm}	5.8 ^{kl}	5.8 ^{kl}	5.8 ^{kl}				
14	87.5 ^{abc}	LY	0.0 ^b	LY	0.0 ^b	LY	0.0 ^e	0.0 ^e	57.5 ^{bc}	0.0 ^f	0.0 ^f	0.0 ^e	85.0 ^{abc}	LY	0.0 ^f	LY	0.0 ^f	0.0 ^e	0.0 ^e	42.5 ^{e-h}	0.0 ^f	89.2 ^{bc}	10.0 ^g	89.2 ^{bc}	0.0 ^b	10.0 ^g	10.0 ^g	0.0 ^b	55.0 ^{bed}	0.0 ^b	55.0 ^{bed}	0.0 ^b				
15	92.5 ^{ab}	W	20.0 ^f	LY	17.5 ^c	LY	0.0 ^e	0.0 ^e	37.5 ^{efg}	10.0 ^e	10.0 ^e	0.0 ^e	87.5 ^{abc}	W	10.0 ^e	LY	10.0 ^e	20.0 ^b	20.0 ^b	32.5 ^{e-h}	20.0 ^d	90.8 ^{bed}	13.3 ^f	0.0 ^b	0.0 ^b	0.0 ^b	0.0 ^b	0.0 ^b	15.8 ^{cd}	13.3 ^g	13.3 ^g	13.3 ^g				
16	75.0 ^{e-f}	R	0.0 ^b	LY	0.0 ^b	LY	0.0 ^e	0.0 ^e	45.0 ^{ef}	20.0 ^d	20.0 ^d	0.0 ^e	95.0 ^{ab}	R	0.0 ^f	LY	0.0 ^f	30.0 ^a	30.0 ^a	37.5 ^{de}	20.0 ^e	85.0 ^{bc}	0.0 ^b	85.0 ^{bc}	0.0 ^b	20.0 ^b	20.0 ^b	0.0 ^b	45.0 ^{e-h}	20.8 ^{cd}	20.8 ^{cd}	20.8 ^{cd}				
17	72.5 ^{ef}	LY	0.0 ^b	LY	0.0 ^b	LY	0.0 ^e	0.0 ^e	0.0 ^j	0.0 ^f	0.0 ^f	0.0 ^e	75.0 ^{de}	LY	0.0 ^f	LY	0.0 ^f	0.0 ^e	0.0 ^e	67.5 ^{de}	20.0 ^d	71.7 ^{gh}	0.0 ^b	71.7 ^{gh}	0.0 ^b	0.0 ^b	0.0 ^b	0.0 ^b	40.0 ^{hk}	10.0 ^h	10.0 ^h	10.0 ^h				
18	65.0 ^{e-h}	Y	0.0 ^b	LY	0.0 ^b	LY	0.0 ^e	0.0 ^e	57.5 ^{bc}	20.0 ^d	20.0 ^d	0.0 ^e	85.0 ^{abc}	Y	0.0 ^f	LY	0.0 ^f	10.0 ^d	20.0 ^b	55.0 ^{de}	0.0 ^f	70.8 ^{gh}	0.0 ^b	70.8 ^{gh}	0.0 ^b	16.7 ^e	16.7 ^e	0.0 ^b	55.8 ^{bc}	14.2 ^{fg}	14.2 ^{fg}	14.2 ^{fg}				
19	55.0 ^{e-j}	W	0.0 ^b	LY	0.0 ^b	LY	0.0 ^e	0.0 ^e	67.5 ^{de}	0.0 ^f	0.0 ^f	0.0 ^e	67.5 ^{de}	W	0.0 ^f	LY	0.0 ^f	0.0 ^e	0.0 ^e	62.5 ^{abc}	20.0 ^e	63.3 ^{bij}	0.0 ^b	63.3 ^{bij}	0.0 ^b	3.3 ^h	3.3 ^h	0.0 ^b	58.3 ^{ab}	10.0 ^h	10.0 ^h	10.0 ^h				
20	77.5 ^{de}	LR	40.0 ^d	LY	10.0 ^d	LY	0.0 ^e	0.0 ^e	32.5 ^{gh}	17.5 ^d	17.5 ^d	0.0 ^e	82.5 ^{bed}	W	30.0 ^{bc}	LY	20.0 ^d	17.5 ^c	17.5 ^c	32.5 ^{gh}	20.0 ^d	81.7 ^{ef}	36.7 ^e	0.0 ^b	0.0 ^b	0.0 ^b	0.0 ^b	0.0 ^b	15.0 ^d	28.3 ^{mm}	19.2 ^{df}	19.2 ^{df}				
21	85.0 ^{bed}	Y	30.0 ^e	LY	30.0 ^e	LY	0.0 ^e	0.0 ^e	35.0 ^{fg}	20.0 ^d	20.0 ^d	0.0 ^e	92.5 ^{ab}	LY	20.0 ^d	LY	20.0 ^d	0.0 ^e	0.0 ^e	30.0 ^{ijk}	30.0 ^b	87.5 ^{de}	30.0 ^b	81.7 ^{ef}	23.3 ^c	3.3 ^h	3.3 ^h	0.0 ^b	32.5 ^{mm}	26.7 ^b	26.7 ^b	26.7 ^b				
22	72.5 ^{ef}	G	17.5 ^f	LY	0.0 ^b	LY	0.0 ^e	0.0 ^e	57.5 ^{bc}	0.0 ^f	0.0 ^f	0.0 ^e	95.0 ^{ab}	LG	30.0 ^{bc}	LY	20.0 ^d	20.0 ^b	20.0 ^b	27.5 ^{e-h}	10.0 ^e	85.0 ^{bc}	28.3 ^d	0.0 ^b	0.0 ^b	0.0 ^b	0.0 ^b	0.0 ^b	10.0 ^f	37.5 ^{kl}	9.2 ^{ab}	9.2 ^{ab}				
23	75.0 ^{e-f}	LY	0.0 ^b	LY	0.0 ^b	LY	0.0 ^e	0.0 ^e	75.0 ^a	0.0 ^f	0.0 ^f	0.0 ^e	85.0 ^{abc}	LY	0.0 ^f	LY	0.0 ^f	0.0 ^e	0.0 ^e	55.0 ^{de}	22.5 ^e	72.5 ^{fg}	0.0 ^b	72.5 ^{fg}	0.0 ^b	0.0 ^b	0.0 ^b	0.0 ^b	64.2 ^a	14.2 ^{fg}	14.2 ^{fg}	14.2 ^{fg}				
24	65.0 ^{e-h}	W	0.0 ^b	LY	0.0 ^b	LY	0.0 ^e	0.0 ^e	47.5 ^{de}	0.0 ^f	0.0 ^f	0.0 ^e	57.5 ^{de}	W	0.0 ^f	LY	0.0 ^f	0.0 ^e	0.0 ^e	45.0 ^{de}	0.0 ^f	61.7 ^{ij}	0.0 ^b	61.7 ^{ij}	0.0 ^b	0.0 ^b	0.0 ^b	0.0 ^b	46.7 ^{e-h}	10.0 ^h	10.0 ^h	10.0 ^h				
25	75.0 ^{e-f}	Y	60.0 ^e	LY	0.0 ^b	LY	0.0 ^e	0.0 ^e	37.5 ^{efg}	0.0 ^f	0.0 ^f	0.0 ^e	95.0 ^{ab}	Y	50.0 ^a	LY	20.0 ^d	10.0 ^d	10.0 ^d	30.0 ^{ijk}	0.0 ^f	85.0 ^{bc}	49.2 ^b	0.0 ^b	0.0 ^b	0.0 ^b	0.0 ^b	0.0 ^b	6.7 ^{ij}	30.8 ^{mm}	6.7 ^{ij}	6.7 ^{ij}				
26	42.5 ^{jk}	LY	0.0 ^b	LY	0.0 ^b	LY	0.0 ^e	0.0 ^e </																												

cotyledon at 39.4% and embryo axis at 34.0%. While the difference between cotyledon and hypocotyl was not statistically significant, both were significantly higher than the embryo axis ($p < 0.01$). Additionally, significant differences were found among the methods used ($p < 0.01$). Direct somatic embryogenesis did not occur in three methods (1, 2, and 36), while successful methods showed DSER rates ranging from 8.3% to 64.2% (Fig. 3). The interaction between explant type and method was also significant ($p < 0.01$). For the embryo axis, DSER rates varied from 10.0% to 75.0%, hypocotyl rates ranged from 5.0% to 67.5%, and cotyledon from 10.0% to 85.0% (Table 3).

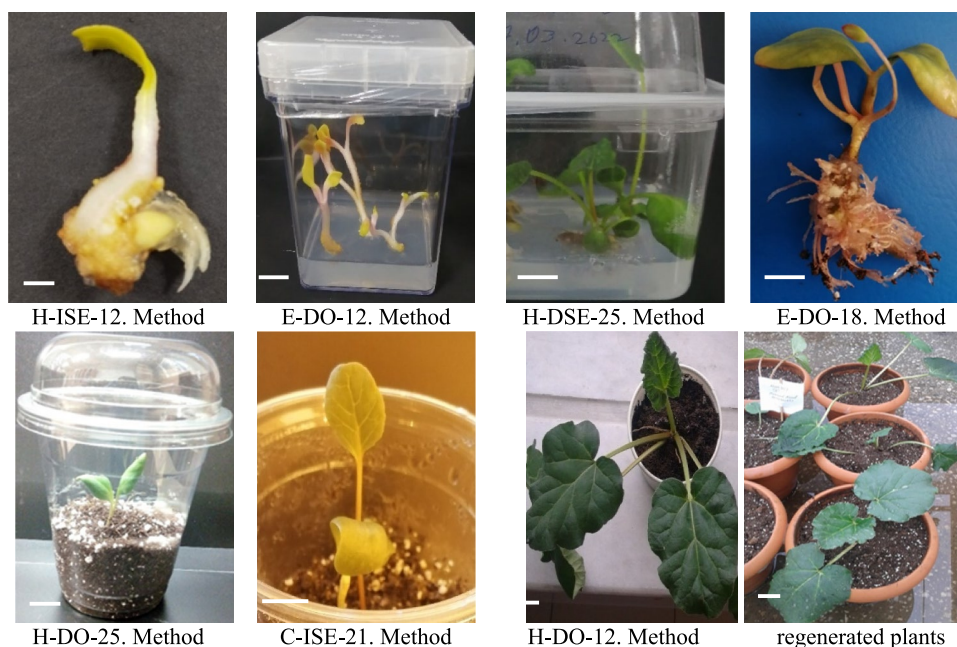
Indirect somatic embryogenesis rate (ISER%) measures the ability to form somatic embryos from calli derived from explants. Indirect somatic embryogenesis rate (ISER) (%) was calculated as follows: $\text{ISER} (\%) = (\text{Number of explants producing indirect somatic embryogenesis} / \text{Total number of explants}) \times 100$. A significant difference in ISER was noted among explant types ($p < 0.01$), with hypocotyl exhibiting the highest rate at 15.3%, followed by cotyledon at 14.7% and embryo axis at 12.2%. The methods used also demonstrated statistically significant differences in ISER ($p < 0.01$). Indirect somatic embryogenesis did not occur in two methods (32 and 34), while successful methods showed ISER rates ranging from 3.3% to 47.5% (Fig. 3). The explant type and method interaction were significant ($p < 0.01$). Specifically, indirect somatic embryogenesis did not occur in 16 methods for the embryo axis, 7 for hypocotyl, and 12 for cotyledon, with methods 32 and 34 showing no occurrence across all explant types. For methods where indirect somatic embryogenesis was successful, ISER rates varied: in the embryo axis from 10.0% to 50.0%;

in hypocotyl from 10.0% to 42.5%; and in cotyledon from 10.0% to 50% (Table 3).

Plant regeneration

The study analyzed plant regeneration through various methods, focusing on the number of plants per explant (PN = Total regenerated plant numbers/explant number) from direct organogenesis (DOPN) and indirect organogenesis (IOPN), as well as direct somatic embryogenesis (DSEPN) and indirect somatic embryogenesis (ISEPN) (Fig. 4). For DOPN, significant differences were observed among explant types ($p < 0.01$), with the embryo axis yielding the highest rate at 0.133, followed by hypocotyl at 0.092 and cotyledon at 0.082. Direct organogenesis did not occur in 23 methods, while successful methods showed PN rates ranging from 0.10 to 1.08. For IOPN, cotyledon had the highest rate of 0.074, with significant differences noted among methods ($p < 0.01$). Indirect organogenesis did not occur in 14 methods, with successful rates between 0.03 and 0.27. In terms of DSEPN, hypocotyl achieved the highest rate at 0.404, followed closely by cotyledon at 0.401 and embryo axis at 0.342, with significant differences among methods ($p < 0.01$). Direct somatic embryogenesis did not occur in three methods, with rates for successful methods varying from 0.08 to 0.64. For ISEPN, hypocotyl again led with a rate of 0.155, followed by cotyledon at 0.147 and embryo axis at 0.117; significant method differences were also noted ($p < 0.01$). Indirect somatic embryogenesis was absent in multiple methods across all explant types. Lastly, the overall number of plants per explant (PPEN) was significantly influenced by explant type ($p < 0.01$), with hypocotyl

Fig. 4 Regenerated plants in different explants and methods (Explant-Regeneration System-Method). C: Cotyledon; H: Hypocotyl. E: Embryo axis. DO: Direct organogenesis. IDO: Indirect organogenesis. DSE: Direct somatic embryogenesis. ISE: Indirect somatic embryogenesis. Explanations of method numbers are given in Table 2. Scale bars = 1 cm



achieving the highest average of 0.717, followed by cotyledon and embryo axis at 0.706 and 0.661, respectively. The PPEN varied between 0.07 and 1.07 across methods, highlighting the variability in plant regeneration efficiency based on explant type and method used (Table 4).

Second stage

Obtainment of callus explant

In all explants used in the first stage, the highest callus formation was achieved with method 30, at 100%. Calluses obtained from embryo axis, hypocotyl, and cotyledon explants cultured for 14 days in the medium and culture conditions of this method [embryo axis-derived callus (E-callus), hypocotyl-derived callus (H-callus), and cotyledon-derived callus (C-callus)] were cultured in 8 different media.

Indirect organogenesis (IOR) and somatic embryogenesis rate (ISER) (%)

The study examined the rates of indirect organogenesis (IOR) and indirect somatic embryogenesis (ISER) across different explant types (Fig. 5). It found significant differences in IOR, with E-callus achieving the highest rate at 28.1%, followed by H-callus at 26.6% and C-callus at 25.9%. The medium also significantly affected IOR, ranging from 0.0% to 47.5%. Indirect organogenesis did not occur in two media (4 and 6), while successful rates varied for each callus type, with E-callus showing rates between 20.0% and 52.5%. For ISER, significant differences were noted among explant types ($p < 0.01$), with C-callus leading at 55.6%, followed by E-callus at 46.3% and H-callus at 42.8%. The media also significantly influenced ISER, with rates from 35.8% to 79.2%. Somatic embryo formation occurred in all media tested, with E-callus rates ranging from 20.0% to 85%, H-callus from 20.0% to 75%, and C-callus from 30.0% to 77.5%. The results showed that the type of explant and the culture medium both significantly influenced the rates of indirect organogenesis and somatic embryogenesis, emphasizing their key role in plant regeneration protocols (Table 5).

Plant regeneration

In the study, plant regeneration via indirect organogenesis (IOPN) and indirect somatic embryogenesis (ISEPN) were investigated and significant differences were revealed according to the explant type. When the IOPN of explants was evaluated according to the average of the media, the

highest IOPN was obtained in E-callus with 0.29, followed by H-callus with 0.28 and H-callus explants with 0.27, respectively, with significant variations in media affecting these rates ($p < 0.01$). In media no. 4 and 6, no plants were formed from indirect organogenesis, while successful media showed IOPN rates ranging from 0.28 to 0.55. In terms of ISEPN, C-callus achieved the highest rate of 0.55, followed by E-callus at 0.47 and H-callus at 0.43, with significant differences among media ($p < 0.01$). Successful media for ISEPN ranged from 0.33 to 0.53. The study also assessed the overall number of plants per explant (PPEN), finding significant effects based on explant type ($p < 0.01$), with C-callus leading at 0.82, followed by E-callus at 0.76 and H-callus at 0.70. PPEN varied between 0.49 and 1.08 across methods (Table 5). The results indicate that both the explant and the medium significantly influence plant regeneration efficiency in indirect organogenesis and somatic embryogenesis.

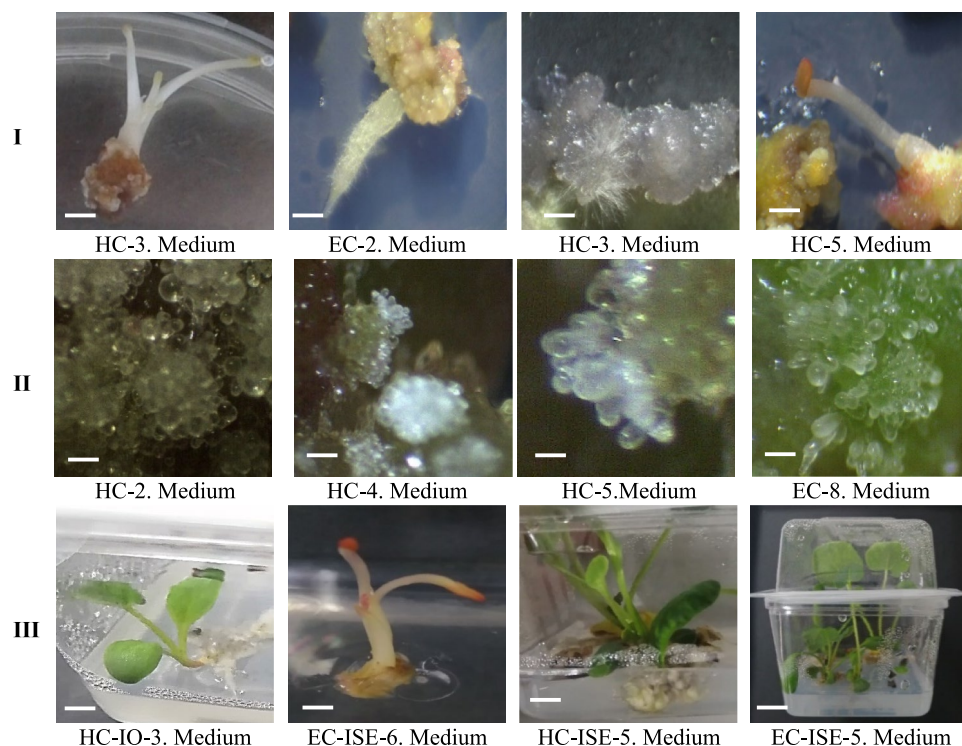
Somaclonal variation

The purpose of micropropagation is to generate many genetically identical plants rapidly. Therefore, in this study, considering the importance of Explant $\times$ Method interactions in the first stage and Explant $\times$ Medium interactions in the second stage in terms of PPEN, the genetic stability of plants obtained from the systems with the highest number of regenerated plants (Table 6) was assessed using the SCoT molecular marker technique. In the study, 30 SCoT primers were tested on the mother plant, and 20 SCoT primers that provided the best amplification from these primers (Table 7) were utilized to assess the genetic stability of regenerated plants grown using 15 different regeneration systems. Gel images of some SCoT primers are given in Fig. 6. An average of 16.65 bands per primer and a total of 333 DNA bands were obtained. The highest number of bands was obtained with the SCoT-1 primer, at 20, while the lowest was observed with the SCoT-22 primer, at 13. When the polymorphism rate (PR%) of the primers was compared, the highest PR% was found in the SCoT-35 primer, at 58.82%, while the lowest was recorded in the SCoT-29 primer, at 5.56%. The average PR% of the primers used in the study was calculated to be 28.37%. It was found that the PIC value of the primers varied between 0.077 (SCoT-21 primer) and 0.110 (SCoT-2 and SCoT-29), and the primers had similar PIC values. On the other hand, SCoT-1 primer had the highest resolving power (R) with 4.125, while SCoT-26 and SCoT-29 primers had the lowest resolving power with 0.250. The discrimination power (D) was determined to vary between 0.282 (SCoT-21 primer) and 0.034 (SCoT-2 primer), with an average of 0.112 across the primers (Table 7).

Table 4 Regenerated plants from direct organogenesis (DOPN), indirect organogenesis (IOPN), direct somatic embryogenesis (DSEPN), indirect somatic embryogenesis (ISEPN) and regenerated plants per explant (PPEP) Plant/Explant) in different explants and methods

Method	Embryo axis						Hypocotyl						Cotyledon						Average					
	DOPN P/Ex	IOPN P/Ex	DSEPN P/Ex	ISEPN P/Ex	PPEN P/Ex	P/Ex	DOPN P/Ex	IOPN P/Ex	DSEPN P/Ex	ISEPN P/Ex	PPEN P/Ex	P/Ex	DOPN P/Ex	IOPN P/Ex	DSEPN P/Ex	ISEPN P/Ex	PPEN P/Ex	P/Ex	DOPN P/Ex	IOPN P/Ex	DSEPN P/Ex	ISEPN P/Ex	PPEN P/Ex	P/Ex
1	0.00 ^h	0.00 ^c	0.00 ^j	0.00 ^e	0.00 ^b	0.00 ^b	0.00 ^g	0.00 ^e	0.00 ^j	0.00 ^d	0.00 ^a	0.00 ^b	0.00 ^g	0.00 ^e	0.00 ^j	0.00 ^d	0.20 ^{cd}	0.20 ^m	0.00 ^h	0.00 ^h	0.00 ^j	0.00 ^b	0.07 ^j	0.07 ^b
2	0.00 ^h	0.00 ^c	0.00 ^j	0.20 ^c	0.20 ^c	0.20 ^c	0.00 ^g	0.00 ^e	0.00 ^j	0.20 ^c	0.20 ^c	0.20 ^{cd}	0.00 ^g	0.30 ^a	0.20 ^j	0.00 ^b	0.20 ^{cd}	0.50 ^{kl}	0.00 ^h	0.00 ^h	0.10 ⁱ	0.00 ^b	0.20 ^d	0.30 ^e
3	0.00 ^h	0.20 ^b	0.10 ^{ij}	0.20 ^c	0.50 ^{lm}	0.00 ^b	0.00 ^g	0.00 ^e	0.00 ^j	0.30 ^b	0.35 ^{kl}	0.00 ^b	0.00 ^g	0.00 ^e	0.10 ^{kl}	0.00 ^b	0.00 ^j	0.10 ^m	0.00 ^h	0.00 ^h	0.07 ^h	0.08 ^e	0.17 ^{ef}	0.32 ^{no}
4	0.20 ^f	0.00 ^c	0.38 ^{efg}	0.20 ^c	0.78 ^{ci}	0.10 ^f	0.00 ^g	0.00 ^e	0.45 ^{cd}	0.20 ^c	0.75 ^{se}	0.00 ^b	0.10 ^f	0.00 ^e	0.53 ^{cde}	0.30 ^b	0.93 ^{abc}	0.00 ^b	0.13 ^f	0.00 ^j	0.00 ^j	0.45 ^{cd}	0.23 ^c	0.82 ^{bce}
5	0.00 ^h	0.10 ^d	0.48 ^{gde}	0.10 ^d	0.68 ^{hij}	0.00 ^b	0.00 ^g	0.20 ^b	0.53 ^{cd}	0.10 ^d	0.83 ^{efg}	0.00 ^b	0.00 ^g	0.00 ^e	0.48 ^{def}	0.00 ^b	0.48 ^{kl}	0.00 ^b	0.48 ^{kl}	0.00 ^h	0.10 ^f	0.49 ^{cd}	0.07 ^{ij}	0.66 ^{gh}
6	0.00 ^h	0.00 ^c	0.20 ^b	0.00 ^c	0.20 ^c	0.20 ^c	0.00 ^g	0.00 ^e	0.20 ^k	0.00 ^c	0.20 ^m	0.00 ^b	0.00 ^g	0.20 ^b	0.18 ^g	0.00 ^b	0.58 ^{gh}	0.00 ^h	0.33 ^{no}	0.07 ^h	0.19 ^e	0.07 ^{ij}	0.33 ^{no}	
7	0.00 ^h	0.20 ^b	0.53 ^{cd}	0.20 ^c	0.93 ^{cde}	0.00 ^b	0.00 ^g	0.10 ^d	0.55 ^{cd}	0.20 ^c	0.85 ^{efg}	0.00 ^b	0.30 ^a	0.30 ^a	0.38 ^{ci}	0.10 ^f	0.78 ^{gh}	0.00 ^h	0.20 ^b	0.00 ^b	0.48 ^g	0.17 ^{ef}	0.85 ^{bce}	
8	0.00 ^h	0.00 ^c	0.38 ^{efg}	0.10 ^d	0.48 ^{lm}	0.00 ^b	0.00 ^g	0.00 ^e	0.43 ^{ci}	0.00 ^c	0.43 ^{kl}	0.00 ^b	0.00 ^g	0.00 ^e	0.45 ^{cd}	0.00 ^b	0.45 ^{kl}	0.00 ^b	0.45 ^{kl}	0.00 ^h	0.03 ^k	0.42 ^{ghi}	0.03 ^k	
9	0.00 ^h	0.10 ^d	0.53 ^{cd}	0.20 ^c	0.83 ^{efg}	0.00 ^b	0.00 ^g	0.10 ^d	0.55 ^{cd}	0.23 ^c	0.88 ^{efg}	0.00 ^b	0.00 ^g	0.10 ^d	0.58 ^{cd}	0.18 ^d	0.85 ^{ef}	0.00 ^h	0.20 ^b	0.10 ^f	0.52 ^{hcd}	0.20 ^d	0.85 ^{bce}	
10	0.30 ^e	0.00 ^c	0.35 ^{efg}	0.00 ^c	0.65 ^{gh}	0.00 ^b	0.00 ^g	0.00 ^e	0.48 ^g	0.20 ^c	0.95 ^{cd}	0.00 ^b	0.10 ^f	0.00 ^e	0.55 ^{cd}	0.20 ^{cd}	0.85 ^{ef}	0.00 ^h	0.23 ^c	0.00 ^j	0.82 ^{bce}	0.13 ^g	0.82 ^{bce}	
11	0.00 ^h	0.00 ^c	0.20 ^b	0.00 ^c	0.20 ^c	0.20 ^c	0.00 ^g	0.00 ^e	0.20 ^k	0.38 ^a	0.58 ^{hi}	0.00 ^b	0.00 ^g	0.00 ^e	0.18 ^g	0.00 ^b	0.38 ⁱ	0.00 ^b	0.00 ^h	0.00 ^h	0.19 ^e	0.19 ^{de}	0.38 ^{mm}	
12	1.08 ^a	0.00 ^c	0.00 ^j	0.00 ^c	1.08 ^{ab}	0.30 ^{cd}	0.00 ^g	0.00 ^e	0.60 ^{cd}	0.18 ^c	1.08 ^{ab}	0.00 ^b	0.20 ^b	0.00 ^c	0.68 ^b	0.00 ^b	0.88 ^b	0.00 ^b	0.53 ^b	0.00 ^h	0.00 ^h	0.43 ^{fi}	0.06 ^{jk}	
13	0.95 ^b	0.00 ^c	0.20 ^b	0.00 ^c	1.15 ^a	0.50 ^b	0.20 ^b	0.00 ^e	0.22 ^k	0.18 ^c	1.10 ^a	0.00 ^b	0.50 ^a	0.00 ^c	0.43 ^{cd}	0.00 ^b	0.93 ^{abc}	0.00 ^h	0.65 ^a	0.07 ^h	0.00 ^h	0.06 ^{jk}	1.06 ^a	
14	0.00 ^h	0.00 ^c	0.58 ^{bce}	0.00 ^c	0.58 ^{bce}	0.00 ^b	0.00 ^g	0.00 ^e	0.65 ^{ab}	0.20 ^c	0.85 ^{efg}	0.30 ^d	0.00 ^c	0.00 ^e	0.43 ^{cd}	0.00 ^b	0.73 ^{gh}	0.10 ^f	0.00 ^h	0.00 ^h	0.55 ^{bed}	0.07 ^{ij}	0.72 ^{efg}	
15	0.20 ^f	0.18 ^c	0.38 ^{efg}	0.10 ^d	0.85 ^{ef}	0.10 ^f	0.10 ^d	0.10 ^d	0.55 ^{cd}	0.10 ^d	0.85 ^{efg}	0.10 ^f	0.20 ^b	0.00 ^c	0.33 ^{cd}	0.20 ^{cd}	0.83 ^{cd}	0.00 ^h	0.13 ^f	0.16 ^d	0.42 ^{ghi}	0.13 ^g	0.84 ^{bce}	
16	0.00 ^h	0.20 ^b	0.45 ^{def}	0.20 ^c	0.85 ^{ef}	0.00 ^b	0.00 ^g	0.30 ^a	0.38 ^g	0.20 ^c	0.88 ^{efg}	0.00 ^b	0.00 ^g	0.10 ^d	0.53 ^{cde}	0.23 ^c	0.85 ^{ef}	0.00 ^h	0.20 ^b	0.00 ^h	0.45 ^{cd}	0.21 ^{cd}	0.86 ^{bce}	
17	0.00 ^h	0.00 ^c	0.00 ^j	0.00 ^c	0.00 ^b	0.00 ^b	0.00 ^g	0.00 ^e	0.68 ^a	0.10 ^d	0.78 ^g	0.00 ^b	0.00 ^g	0.00 ^e	0.53 ^{cde}	0.20 ^{cd}	0.73 ^{gh}	0.00 ^h	0.00 ^h	0.00 ^h	0.40 ^{hij}	0.10 ^h	0.50 ^{kl}	
18	0.00 ^h	0.20 ^b	0.58 ^{bce}	0.20 ^c	0.98 ^{bcd}	0.00 ^b	0.00 ^g	0.10 ^d	0.55 ^{cd}	0.23 ^c	0.88 ^{efg}	0.00 ^b	0.00 ^g	0.20 ^b	0.55 ^{cd}	0.00 ^b	0.75 ^{cd}	0.00 ^h	0.00 ^h	0.17 ^d	0.56 ^{bce}	0.14 ^g	0.87 ^g	
19	0.00 ^h	0.00 ^c	0.68 ^b	0.00 ^c	0.68 ^b	0.00 ^b	0.00 ^g	0.10 ^d	0.63 ^{abc}	0.20 ^c	0.93 ^{cd}	0.00 ^b	0.00 ^g	0.00 ^e	0.45 ^{cd}	0.10 ^f	0.55 ^{gh}	0.00 ^h	0.00 ^h	0.03 ⁱ	0.58 ^{ab}	0.10 ^h	0.72 ^{efg}	
20	0.40 ^d	0.10 ^d	0.30 ^{gh}	0.18 ^c	1.08 ^{ab}	0.30 ^{cd}	0.23 ^b	0.30 ^{cd}	0.40 ^{cd}	0.20 ^c	1.10 ^a	0.00 ^b	0.40 ^b	0.20 ^b	0.10 ^d	0.25 ^{ij}	1.03 ^a	0.90 ^{cd}	0.23 ^c	0.18 ^c	0.32 ^{kl}	0.19 ^{de}	1.07 ^a	
21	0.30 ^e	0.00 ^c	0.35 ^{efg}	0.20 ^c	0.85 ^{ef}	0.20 ^c	0.00 ^g	0.00 ^e	0.30 ^k	0.30 ^b	0.80 ^{efg}	0.00 ^b	0.00 ^g	0.10 ^d	0.28 ^{ij}	0.10 ^f	0.85 ^{ef}	0.00 ^h	0.28 ^d	0.03 ⁱ	0.32 ^{kl}	0.27 ^d	0.85 ^{bce}	
22	0.20 ^f	0.00 ^c	0.58 ^{bce}	0.00 ^c	0.75 ^{ci}	0.30 ^{cd}	0.20 ^b	0.20 ^b	0.28 ^{jk}	0.18 ^c	0.95 ^{cd}	0.00 ^b	0.38 ^e	0.10 ^d	0.28 ^{ij}	0.10 ^f	0.85 ^{ef}	0.00 ^h	0.28 ^d	0.10 ^f	0.38 ^{gh}	0.09 ^{hi}	0.85 ^{bce}	
23	0.00 ^h	0.00 ^c	0.75 ^a	0.00 ^c	0.75 ^{ci}	0.00 ^b	0.00 ^g	0.00 ^e	0.55 ^{cd}	0.23 ^c	0.78 ^g	0.00 ^b	0.00 ^g	0.00 ^e	0.63 ^{bce}	0.20 ^{cd}	0.83 ^{cd}	0.00 ^h	0.00 ^h	0.00 ^h	0.64 ^a	0.14 ^g	0.78 ^{gde}	
24	0.00 ^h	0.00 ^c	0.48 ^{bde}	0.00 ^c	0.48 ^{bde}	0.00 ^b	0.00 ^g	0.00 ^e	0.45 ^{cd}	0.00 ^c	0.45 ^{kl}	0.00 ^b	0.00 ^g	0.00 ^e	0.48 ^{bce}	0.30 ^b	0.78 ^{gh}	0.00 ^h	0.00 ^h	0.00 ^h	0.47 ^{cd}	0.10 ^h	0.57 ^{gh}	
25	0.60 ^e	0.00 ^c	0.45 ^{def}	0.00 ^c	1.05 ^{abc}	0.60 ^a	0.15 ^c	0.30 ^k	0.30 ^k	0.00 ^c	1.05 ^{abc}	0.40 ^b	0.10 ^d	0.00 ^b	0.28 ^{ij}	0.20 ^{cd}	0.98 ^{ab}	0.00 ^h	0.53 ^b	0.08 ^e	0.34 ^{kl}	0.07 ^{ij}	1.03 ^a	
26	0.00 ^h	0.00 ^c	0.35 ^{efg}	0.00 ^c	0.35 ⁱ	0.00 ^b	0.00 ^g	0.00 ^e	0.43 ^{ci}	0.10 ^d	0.53 ^{hij}	0.00 ^b	0.00 ^g	0.00 ^e	0.48 ^{bce}	0.10 ^f	0.58 ^{gh}	0.00 ^h	0.00 ^h	0.00 ^h	0.42 ^{ghi}	0.07 ^{ij}	0.48 ⁱ	
27	0.20 ^f	0.00 ^c	0.20 ^b	0.00 ^c	0.40 ^{mm}	0.10 ^f	0.20 ^b	0.20 ^b	0.38 ^g	0.20 ^c	0.88 ^{efg}	0.00 ^b	0.10 ^d	0.00 ^b	0.43 ^{cd}	0.30 ^b	0.93 ^{abc}	0.13 ^f	0.10 ^f	0.00 ^h	0.33 ^{kl}	0.17 ^{ef}	0.73 ^{def}	
28	0.30 ^e	0.20 ^b	0.10 ^j	0.20 ^c	0.80 ^{cd}	0.33 ^c	0.10 ^d	0.10 ^d	0.23 ^k	0.20 ^c	0.85 ^{efg}	0.10 ^f	0.20 ^b	0.00 ^b	0.33 ^{ghi}	0.30 ^b	0.93 ^{abc}	0.24 ^e	0.17 ^d	0.00 ^h	0.22 ^{mm}	0.23 ^c	0.86 ^{bce}	
29	0.10 ^g	0.00 ^c	0.53 ^{cd}	0.30 ^b	0.93 ^{cde}	0.20 ^c	0.00 ^g	0.00 ^e	0.55 ^{cd}	0.10 ^d	0.85 ^{efg}	0.10 ^f	0.00 ^c	0.00 ^b	0.65 ^{bce}	0.00 ^b	0.75 ^{cd}	0.00 ^h	0.00 ^h	0.00 ^h	0.58 ^{ab}	0.13 ^g	0.84 ^{bce}	
30	0.00 ^h	0.10 ^d	0.00 ^b	0.40 ^b	0.50 ^{lm}																			

Fig. 5 I: Indirect organogenesis in different explants and media (Explant-Medium). **II:** Indirect somatic embryogenesis. **III:** Regenerated plants. EC: Embryo axis-callus; HC: Hypocotyl-callus. IO: indirect organogenesis. ISE: indirect somatic embryogenesis. Explanations of medium numbers are given in Table 2. Scale bars: I, III = 1 cm; II = 0.5 mm



SCoT band patterns generally showed acceptable polymorphism. The genetic relationship between the systems was revealed by a dendrogram obtained with NTSYS pc 2.1 software based on Jaccard similarity coefficient and UPGMA algorithm (Fig. 7). According to the research results, the similarity coefficients between micropropagation systems varied between 0.845–0.991. Moreover, the similarity coefficients between regenerated plants from different systems and the mother plant varied between 0.904 and 0.991 (Table 8). The highest similarity to the mother plant was determined with 0.991 in the system obtained by culturing the hypocotyl explant in the 12th method (system no. 6). This was followed by 0.972 in 13, 0.960 in 4 and other systems (Table 8). The lowest similarity to the mother plant was determined with 0.904 in regeneration system no. 15. Moreover, it was determined that these similarities obtained with the mother plant were at an acceptable level in terms of micropropagation.

Discussion

Medicinal and aromatic plants have been extensively used for treating various diseases, with bioactive compounds demonstrating significant pharmacological effects (Bernela et al. 2023; Pandith and Khan 2022). The increasing demand for these plants necessitates alternative production techniques to prevent habitat degradation and ensure the survival of endangered species (Mehalaine and Chenchouni

2021). Tissue culture is crucial for in vitro propagation, regeneration, and conservation of rare medicinal plants (Das and Rout 2002). Niazi (2019) reported the successful use of plant tissue culture methods for overproduction of bioactive compounds, in vitro preservation and micropropagation of medicinal plants. In vitro micropropagation allows rapid propagation and consistent high-quality production (Mehalaine and Chenchouni 2021; Pandith et al. 2018). In wild plants, reasons such as unsuitable environmental conditions and seed dormancy during seed germination reduce the germination rate and, as a result, prevent the ex-situ preservation of plants (Yamaner and Erdağ 2021).

In vitro germination offers a solution for wild plants that are difficult to germinate using traditional methods. In this study, the highest germination rate and the minimum average germination time in both explants were found in $\frac{1}{2}$ MS medium containing 1.75 mg/l GA3 and 1 mg/l IBA. GA3 stimulates seed germination by inducing hydrolytic enzymes, weakening the protective tissues, the endosperm or seed coat, and promoting the growth of the embryo by providing mobility of seed storage reserves (Banaszczyk et al. 2025; Kucera et al. 2005). On the other hand, methods such as cold moist stratification, GA3 application, mechanical abrasion of the seed coat, and cutting of the seed tip are used to break seed dormancy (Keskiner and Tuncer 2019; Rahmanpour et al. 2005). In plant tissue culture, browning refers to a phenomenon in which explants release brown substances, or phenolics, from their own tissues into the medium during the process of differentiation and/or

Table 5 Indirect organogenesis formation (IOR %), indirect somatic embryogenesis formation (ISER %), regenerated plants from indirect organogenesis (IOPN), regenerated plants from indirect somatic embryogenesis (ISEPN), and regenerated plants per explant (PPEN) (Plant/Explant) in different explants and media

Medium	E-callus						H-callus						C-callus						Average	
	IOR	ISER	IOPN	ISEPN	PPEN		IOR	ISER	IOPN	ISEPN	PPEN		IOR	ISER	IOPN	ISEPN	PPEN		IOR	
1	40.0 ^b	32.5 ^e	0.40 ^b	0.30 ^d	0.70 ^{cd}		37.5 ^b	20.0 ^e	0.38 ^b	0.20 ^e	0.58 ^e		40.0 ^b	55.0 ^e	0.40 ^b	0.50 ^d	0.90 ^b		39.2 ^b	35.8 ^d
2	27.5 ^c	45.0 ^b	0.28 ^c	0.45 ^{bc}	0.73 ^{cd}		27.5 ^c	52.5 ^b	0.28 ^d	0.53 ^b	0.80 ^b		27.5 ^c	52.5 ^c	0.28 ^d	0.53 ^{cd}	0.80 ^{bc}		27.5 ^{cd}	50.0 ^b
3	57.5 ^a	20.0 ^d	0.58 ^a	0.20 ^f	0.78 ^{bc}		37.5 ^b	45.0 ^{bc}	0.38 ^b	0.45 ^{bc}	0.83 ^b		20.0 ^d	62.5 ^{bc}	0.20 ^d	0.63 ^{bc}	0.83 ^{bc}		38.3 ^b	42.5 ^c
4	0.0 ^d	52.5 ^b	0.00 ^e	0.53 ^b	0.53 ^e		0.0 ^e	32.5 ^d	0.00 ^e	0.33 ^d	0.33 ^d		0.0 ^e	62.5 ^{bc}	0.00 ^e	0.63 ^{bc}	0.63 ^d		0.0 ^e	49.2 ^b
5	52.5 ^a	45.0 ^b	0.60 ^a	0.50 ^{bc}	1.10 ^a		60.0 ^a	40.0 ^{cd}	0.68 ^a	0.40 ^{cd}	1.08 ^a		30.0 ^e	67.5 ^{ab}	0.38 ^b	0.68 ^{ab}	1.06 ^a		47.5 ^a	50.8 ^b
6	0.0 ^d	85.0 ^a	0.00 ^e	0.85 ^a	0.85 ^b		0.0 ^e	75.0 ^a	0.00 ^e	0.75 ^a	0.75 ^b		0.0 ^e	77.5 ^a	0.00 ^e	0.78 ^a	0.78 ^c		0.0 ^e	79.2 ^a
7	27.5 ^c	47.5 ^b	0.28 ^c	0.48 ^{bc}	0.75 ^{bc}		20.0 ^d	32.5 ^d	0.20 ^d	0.33 ^d	0.53 ^c		40.0 ^b	37.5 ^d	0.40 ^b	0.38 ^e	0.78 ^c		29.2 ^d	39.2 ^{cd}
8	20.0 ^c	42.5 ^{bc}	0.20 ^d	0.43 ^c	0.63 ^{de}		30.0 ^e	45.0 ^{bc}	0.30 ^{bc}	0.45 ^{bc}	0.75 ^b		50.0 ^a	30.0 ^d	0.50 ^a	0.30 ^e	0.80 ^{bc}		33.3 ^c	39.2 ^{cd}
Average	28.1 ^A	46.3 ^B	0.29 ^A	0.47 ^B	0.76 ^B		26.6 ^A	42.8 ^C	0.28 ^{AB}	0.43 ^C	0.70 ^C		25.9 ^B	55.6 ^A	0.27 ^B	0.55 ^A	0.82 ^A		4.87 ^{**}	60.49 ^{**}
F value (Explant-E)	148.43 ^{**}	55.71 ^{**}	223.19 ^{**}	81.45 ^{**}	55.46 ^{**}		172.14 ^{**}	49.12 ^{**}	154.29 ^{**}	49.12 ^{**}	63.36 ^{**}		434.14 ^{**}	42.62 ^{**}	227.14 ^{**}	52.95 ^{**}	23.36 ^{**}		452.50 ^{**}	97.17 ^{**}
F value (Medium-M)																				
F value (E × M)																			63.30 ^{**}	25.44 ^{**}
CV (%)	12.57	10.81	10.54	9.04	6.04		11.53	10.92	12.86	10.92	8.08		6.82	8.60	9.30	7.87	6.11			

Differences between averages shown with the same lower-case letter in the same column and the same upper-case letter in the same row are insignificant ($p > 0.05$). P/Ex: Plant/Explant. E-callus: Embryo axis-callus; H-callus: Hypocotyl-callus. C-callus: Cotyledon-callus. **: Significant at the 0.01 level

redifferentiation (Amente and Chimdessa 2021; Shen et al. 2005). Phenolic compounds are typically found in healthy plant tissues but can increase in response to tissue injury or stress (Pratyusha 2022; Onuoha et al. 2011). Moreover, browning may occur in the explants due to the oxidation of polyphenolic compounds released from the cut ends of explants with high phenolic content, posing a significant problem in the plant regeneration process (Devi et al. 2024). To get rid of this problem, some procedures can be used such as the use of antioxidant chemicals like ascorbic acid, PVP (Singh and Chaturvedi 2022; Satheesh and Sridharan 2014; Patil et al. 2011) and activated carbon (Kumar et al. 2019) as well as subculturing of explants at regular intervals (Desai et al. 2018). The effectiveness of these procedures varies from species to species and according to the physiological conditions of the plant. In this study, we found that using 0.7 mg/l PVP in culture media and culturing the explants in the dark for 14 days and then subculturing them in the light for 21 days was the most effective method to control browning. This method was successfully employed in prior studies to avoid browning in *Punica granatum* L. (Verma et al. 2021). PVP, a synthetic polymer, acts as a phenolic adsorbent. It binds phenolic compounds via hydrogen bonding and prevents their oxidation by scavenging free radicals and reactive intermediates. This action helps maintain the physiological integrity of the explant cells (Permadi et al. 2024).

Auxins and cytokinins play important roles in cell growth and differentiation, callus formation, and adventitious shoot and root formation from callus in plant tissue culture (Duangporn and Siripong 2009). In the current study, the highest callus formation rate (100%) was found in all explant types by using equal amounts of auxin (1 mg/l 2,4-D) and cytokinin (0.5 mg/l BAP + 0.5 mg/l KIN) in method 30. Using equal ratios of auxin/cytokinin increased callus induction in other *Rheum* species and similar results were obtained (Khan et al. 2022; Tuncer and Günsan 2017). In this study, callus formation rates varied according to the explant types and methods, it was determined that hypocotyl and cotyledon explants had higher values than the embryo axis. Previous research reported that the highest callus formation (58.3%) was obtained from leaf explants of *Rheum franzenbachii* Munt. in MS + 2 mg/l BAP + 0.5 mg/l NAA (Wang et al. 2011). Moreover, many studies in *Rheum* have concluded that hypocotyl explants are more successful for callus formation (Tuncer and Günsan 2017; Farzami Tabin et al. 2016; Farzami Sepehr and Ghorbanli 2002). Besides, the use of auxin types of alone significantly increased callus formation in a study on *Rheum coreanum* Nakai (Mun and Mun 2016), while 0.5 mg/l 2,4-D caused 100% callus formation in *Rheum webbianum* Royle (Rashid et al. 2014). Further, calli developing in vitro vary in color depending on the plant species, explant type and nutrient content (Winarto et

Table 6 Regeneration systems used in somaclonal variation

Regeneration System no	Explant	Method/Medium	EBBS	DOPN	IOPN	DSEPN	ISEPN
2	Embryo axis	13	1.15	0.95	0.00	0.20	0.00
3	Hypocotyl	13	1.10	0.50	0.20	0.22	0.18
4	Hypocotyl	20	1.10	0.30	0.20	0.40	0.20
5	Embryo axis	12	1.08	1.08	0.00	0.00	0.00
6	Hypocotyl	12	1.08	0.30	0.00	0.60	0.18
7	Embryo axis	20	1.08	0.40	0.10	0.40	0.18
8	Embryo axis	25	1.05	0.60	0.00	0.45	0.00
9	Hypocotyl	25	1.05	0.60	0.15	0.30	0.00
10	Cotyledon	20	1.03	0.40	0.18	0.25	0.20
11	Embryo axis	33	0.98	0.00	0.10	0.58	0.30
12	Cotyledon	25	0.98	0.40	0.10	0.28	0.20
13	Embryo axis	18	0.98	0.00	0.20	0.58	0.20
14	E-Callus	5. Medium	1.10	—	0.60	—	0.50
15	H-Callus	5. Medium	1.08	—	0.68	—	0.40
16	C-Callus	5. Medium	1.06	—	0.38	—	0.68

Table 7 Band information and polymorphism rate of SCoT primers used in the study

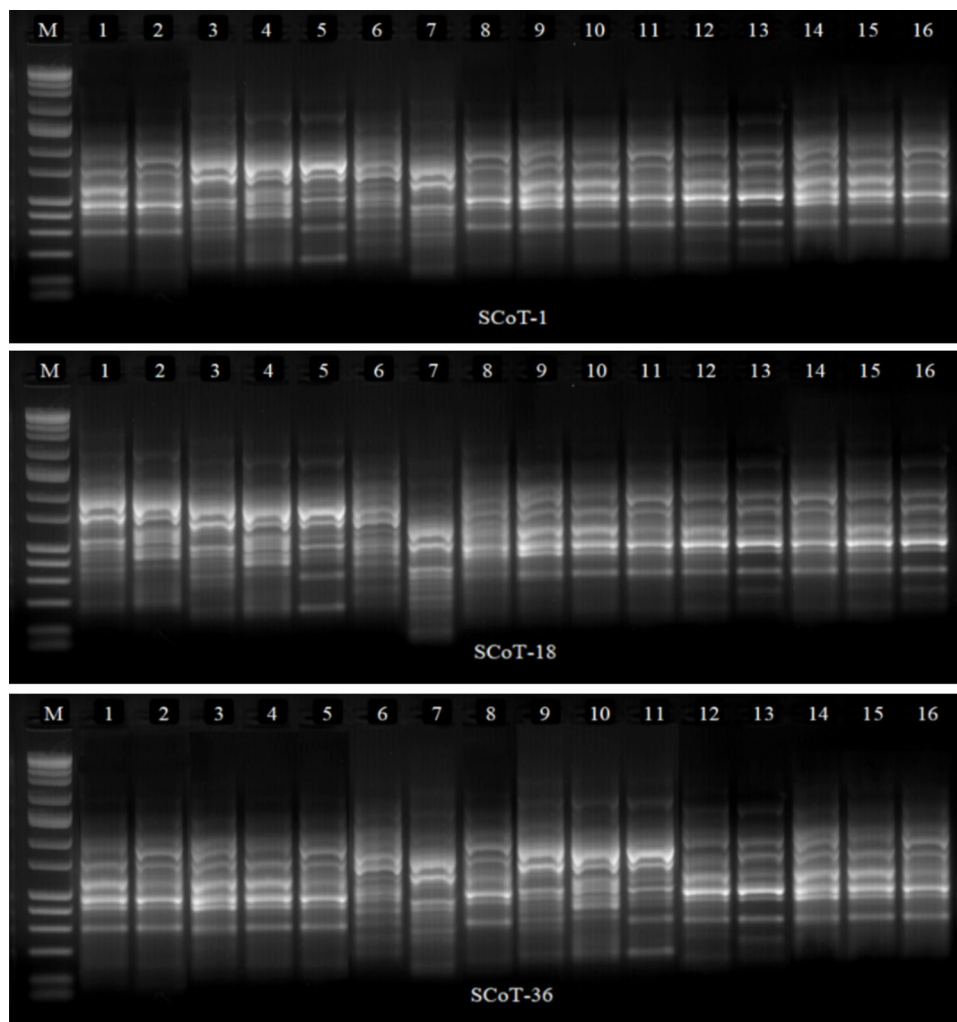
Primer	PIC ^a	D ^b	R ^c	TB ^d	MB ^e	PB ^f	P (%) ^g
SCoT-1	0.091	0.207	4.125	20	9	11	55.00
SCoT-2	0.110	0.034	0.625	18	16	2	11.11
SCoT-4	0.108	0.065	1.250	19	15	4	21.05
SCoT-5	0.109	0.051	0.875	17	16	1	5.88
SCoT-6	0.109	0.058	0.875	15	11	4	26.67
SCoT-8	0.106	0.098	1.500	15	12	3	20.00
SCoT-10	0.106	0.092	1.500	16	13	3	18.75
SCoT-12	0.095	0.185	3.500	18	12	6	33.33
SCoT-18	0.086	0.235	3.750	15	8	7	46.67
SCoT-21	0.077	0.282	2.625	16	9	7	43.75
SCoT-22	0.099	0.157	2.125	13	9	4	30.77
SCoT-25	0.108	0.062	0.875	14	10	4	28.57
SCoT-26	0.110	0.017	0.250	15	13	2	13.33
SCoT-28	0.104	0.115	2.125	18	12	6	33.33
SCoT-29	0.110	0.014	0.250	18	17	1	5.56
SCoT-30	0.109	0.048	0.875	18	16	2	11.11
SCoT-34	0.107	0.077	1.250	16	13	3	18.75
SCoT-35	0.098	0.162	2.875	17	7	10	58.82
SCoT-36	0.098	0.164	3.250	19	10	9	47.37
SCoT-40	0.103	0.121	2.000	16	10	6	37.50
Average	0.102	0.112	1.825	16.65	11.90	4.75	28.37
Total	—	—	—	333	238	95	

^aPolymorphic information content (Liu 1998). ^bDiscriminatory power (Tessier et al. 1999). ^cResolving power (Prevost and Wilkinson 1999). ^dTotal number of bands. ^eMonomorphic bands. ^fPolymorphic bands. ^gPercentage polymorphism (%)

al. 2011). In this study, different colors were observed in the calli obtained. Moreover, it was observed that each of these calli had different regeneration capacities. In our findings, it was determined that the calli developing in method 30 were light green and green in color and that these calli had high regeneration efficiency. Callus color influences regeneration capacity, with green to light green exhibiting high potential, while red yellow calli shows the lowest efficiency (Jia et al. 2014; Winarto et al. 2011). A study on *Ruta chalepensis* found that yellowish green calli had high regeneration rates

(approximately 98.4%), whereas brown calli showed lower regeneration capacity and had lower phenolic compound content (Qahtan et al. 2022). Also, studies have shown that callus browning is often associated with elevated levels of phenolic compounds and reactive oxygen species (ROS), which can impair cellular viability and regeneration potential (He et al. 2009; Khosroushahi et al. 2011; Rao et al. 2015). For instance, research on *Paeonia ostii* revealed that browned calli exhibited a significant accumulation of phenolic compounds, particularly flavonoids such as luteolin

Fig. 6 Gel electrophoresis images obtained from some SCoT primers. M: Marker; 1: Donor plant. Explanations of systems 2–16 are given in Table 6



and diosmetin. These compounds are known to contribute to oxidative stress, which can lead to cell death and reduced regenerative capacity (Wen et al. 2025).

Plant regeneration depends on two basic systems: organogenesis and somatic embryogenesis (Bhatia and Bera 2015). The capacity for shoot and root regeneration via somatic embryogenesis and organogenesis varies depending on the culture medium components, plant species, and explant type. Plant growth regulators, especially auxin and cytokinin types, play a significant role in plant regeneration (Hill and Schaller 2013). The combination of auxins and cytokinins enhances regeneration by activating signaling pathways that lead to increased organogenic responses (Pernisová et al. 2009; Verma et al. 2021). However, Khan et al. (2022) reported that high cytokinin (BAP and KIN) concentrations provided direct rooting from callus in *R. spiciforme*, whereas GA3 application, together with high cytokinin, increased plant regeneration by providing shoot formation.

In our study, different regeneration systems (organogenesis and somatic embryogenesis) were observed in different

explants in the same medium and even on the same explant. Similarly, Ouyang et al. (2016) observed both organogenesis and somatic embryogenesis systems in the same medium in *Metabriggsia ovalifolia*. The highest direct organogenesis rates were recorded in MS medium with 3.0 mg/l BAP + 1.0 mg/l NAA (method 12) and 3.0 mg/l BAP + 0.2 mg/l NAA + 1.0 mg/l GA3 (method 13) under light conditions. Conversely, the highest indirect organogenesis rate occurred in MS medium containing 1.0 mg/l BAP + 1.0 mg/l KIN + 1.0 mg/l 2,4-D (method 36) in dark conditions. These results showed that media containing high amounts of cytokinin and light conditions promoted organogenesis directly, while media containing equal amounts of auxin and cytokinin and dark conditions promoted organogenesis through callus formation. Some studies have shown that equal concentrations of cytokinin and auxin enhance shoot regeneration, as seen in *Rheum emodi* with 15 μ M BAP and 15 μ M IBA (Tabin et al. 2018) and in *Rheum spiciforme* with 15 μ M BAP and 15 μ M 2,4-D (Tabin et al. 2016). Additionally, in our study, the direct and indirect organogenesis rates of explants varied according to the medium composition. This situation can be

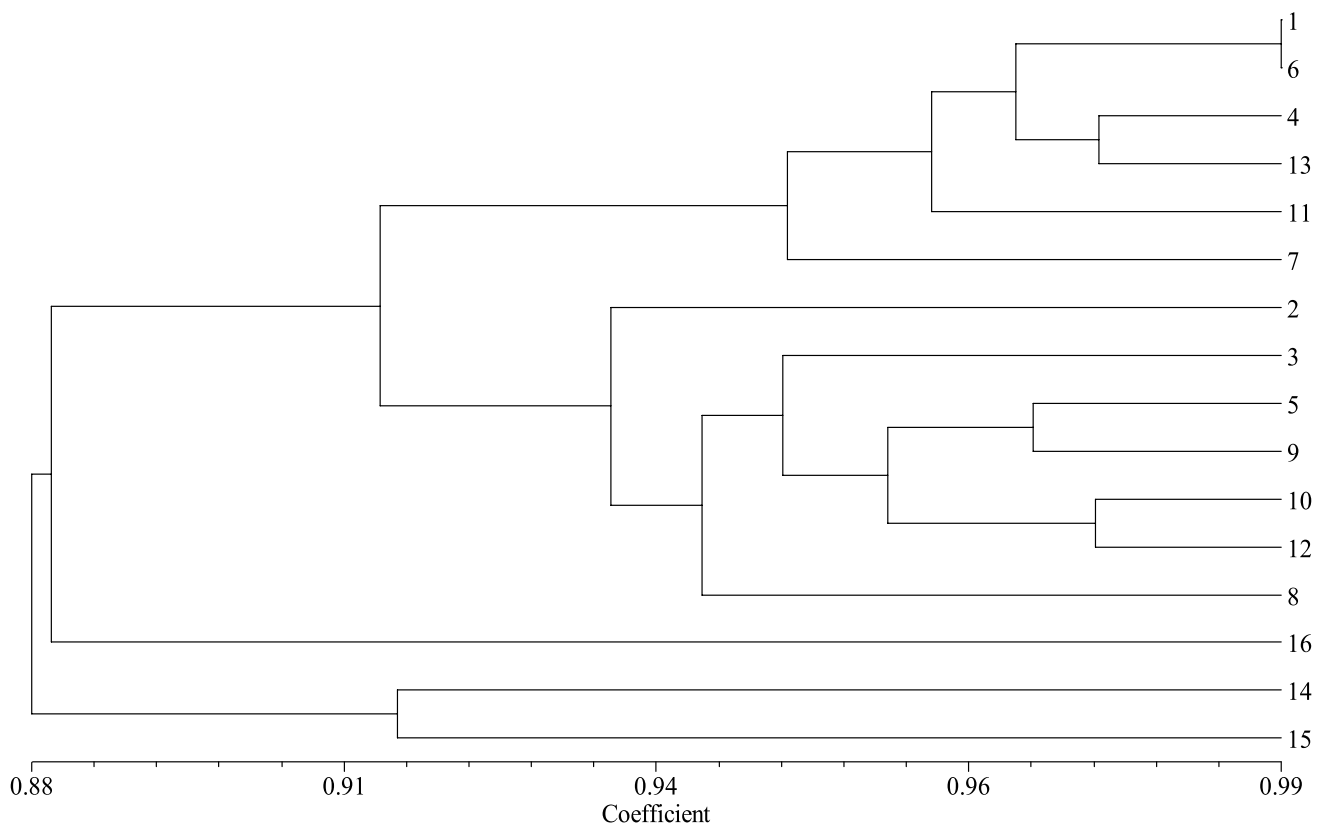


Fig. 7 Dendrogram of genetic relationship between donor plant (1) and regeneration systems

explained by the endogenous hormone levels of the explants used in the study. Previous findings highlight the influence of tissue-specific hormonal profiles on callus formation and plant regeneration (Liang et al. 2022). Furthermore, plant morphogenesis is entirely controlled by endogenous hormones, while exogenous hormones may only operate as modulators of their effects. Recent evidence highlights that plant morphogenesis is regulated primarily by endogenous hormone biosynthesis and distribution, especially in the case of auxins, which establish spatial and temporal gradients essential for cell fate determination (TAA1/IPA/YUCCA pathway). Exogenous auxins and cytokinins, when applied in plant tissue culture, act not as direct inducers of morphogenesis but as modulators that can influence or reprogram endogenous hormone biosynthesis, transport, and canalization (Pasternak and Steinmacher 2024).

On the other hand, cytokinin-type plant growth regulators, although they have a high effect on shoot formation, are insufficient in some plant species. In this regard, it has been reported in more than one study (Hesami et al. 2018; Thakur et al. 2018; Xiao et al. 2016) that the use of GA₃, together with cytokinin derivatives, increased shoot formation and multiplication. In this study, the highest direct somatic embryogenesis rate occurred in light MS + 4.0 mg/l BAP + 1.0 mg/l 2,4-D (method 23) and in dark conditions

in MS + 2.0 mg/l BAP + 1.0 mg/l 2,4-D (method 33) and 2.0 mg/l BAP + 2.0 mg/l 2,4-D (method 34). The highest indirect somatic embryogenesis rate occurred in dark conditions in MS medium containing 0.5 mg/l BAP + 0.5 mg/l KIN + 1.0 mg/l 2,4-D (method 30). High cytokinin concentrations were effective for inducing somatic embryogenesis, as reported by Lal and Ahuja (1989) in *R. emodi* using 10 μ M BAP + 5 μ M IBA, and Khan et al. (2022) noted similar results with 1 μ M NAA + 25 μ M BAP in *R. spiciforme*. Somatic embryogenesis has also been successfully applied to other medicinal plants like *Boswellia serrata* (Naikawadi et al. 2025) and *Polygonatum cyrtonema* (Yang et al. 2024). Moreover, cytokinin responsiveness is itself dependent on the cell's ability to produce endogenous auxin via the TAA1 pathway. The presence of ARR1 binding sites in the TAA1 promoter region provides a direct regulatory link between cytokinin signaling and auxin biosynthesis. Therefore, the exogenous application of cytokinins can indirectly influence auxin levels and distribution, further shaping the morphogenetic trajectory (callus formation, organogenesis, or embryogenesis) (Pasternak and Steinmacher 2024).

In the second stage of this study, 14-day-old calli obtained from embryo axis (E-callus), hypocotyl (H-callus), and cotyledon (C-callus) explants using method 30 from the first stage were used for indirect organogenesis and indirect

Table 8 Genetic similarity values between 15 methods/media and control determined with 20 SCoT primers using Jaccard Similarity Coefficient

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	1.000															
2	0.940	1.000														
3	0.934	0.939	1.000													
4	0.960	0.901	0.895	1.000												
5	0.958	0.945	0.939	0.919	1.000											
6	0.991	0.931	0.925	0.969	0.949	1.000										
7	0.954	0.906	0.900	0.944	0.924	0.951	1.000									
8	0.927	0.926	0.932	0.888	0.945	0.918	0.894	1.000								
9	0.946	0.933	0.945	0.912	0.969	0.937	0.912	0.944	1.000							
10	0.928	0.927	0.950	0.889	0.957	0.919	0.900	0.950	0.969	1.000						
11	0.951	0.909	0.891	0.965	0.927	0.960	0.941	0.896	0.903	0.891	1.000					
12	0.922	0.926	0.956	0.888	0.945	0.913	0.894	0.932	0.957	0.975	0.885	1.000				
13	0.972	0.913	0.907	0.975	0.937	0.969	0.950	0.900	0.919	0.901	0.966	0.895	1.000			
14	0.911	0.858	0.846	0.889	0.876	0.914	0.871	0.868	0.864	0.852	0.885	0.845	0.890	1.000		
15	0.904	0.879	0.896	0.864	0.915	0.895	0.870	0.907	0.902	0.907	0.867	0.901	0.877	0.914	1.000	
16	0.911	0.858	0.863	0.889	0.888	0.914	0.894	0.868	0.881	0.869	0.891	0.868	0.901	0.886	0.873	1.000

1: Donor plant. Explanations of systems, 2–16 are given in Table 6

somatic embryogenesis. According to the average medium, the highest ISER was obtained in C-callus, with 55.6%, while the highest IOR was obtained in E-callus, with 28.1%. The observed differences in ISER and IOR between the C-callus and E-callus types may be attributed to the intrinsic physiological and genetic characteristics of the explant sources from which they originated, as well as their differential responsiveness to in vitro culture conditions (Esposito-Polesi et al. 2020). The highest indirect organogenesis occurred in MS + 2.0 mg/l BAP + 2.0 mg/l KIN + 0.2 mg/l NAA + 1.0 mg/l GA3 (medium 5), while the highest indirect somatic embryogenesis occurred in MS + 2.0 mg/l BAP + 2.0 mg/l KIN + 1.0 mg/l GA3 (medium 6). Similarly in previous studies on other plant species like *Coccinia cordifolia* (Sarkar and Alam 2022) and *Alocasia amazonica* (Raju et al. 2022) showed high indirect organogenesis with similar growth regulator concentrations. Furthermore, dark incubation was crucial for successful organogenesis and somatic embryogenesis, compatible with studies showing light's inhibitory effect on certain gene expressions related to biosynthesis (Devlin et al. 2003; Cavallaro et al. 2022). The dark conditions promote indirect regeneration by modulating auxin biosynthesis and signaling pathways. Specifically, darkness facilitates the upregulation of auxin biosynthesis genes such as YUCCA and TAA1, as well as auxin response factors like ARF7 and ARF19. These genes are crucial for callus formation and subsequent organogenesis (Luo and Di 2023; Xie et al. 2021). Furthermore, the WOX11–LBD16 signaling pathway has been identified as a key regulator. WOX11 activates LBD16, which, in turn, promotes cell division and the establishment of a root primordium-like identity in callus cells, thereby facilitating shoot regeneration. This pathway is particularly active under dark conditions, aligning with the enhanced regeneration observed in the absence of light (Liu et al. 2018). According to the data from the first and second stages of our research, 15 methods/media with the highest number of plants obtained from four different regeneration systems (DOPN, IOPN, DSEPN, and ISEPN) in terms of the number of plants per explant were determined. The highest number of plants per explant (PPEN) was 1.15 plants/explant, obtained from embryo axis cultures using method 13 (3 mg/l BAP, 0.2 mg/l NAA, 1.0 mg/l GA3). Similar high regeneration was observed in hypocotyl explants using methods 13 and 20 (4 mg/l KIN, 0.2 mg/l NAA, 1.0 mg/l GA3). These results are consistent with prior studies on *Rheum* species, where shoot formation was high with BAP and IBA combinations (Tuncer and Günsan 2017; Malik et al. 2010). Higher cytokinin and lower auxin concentrations were key for shoot formation (Pokimica et al. 2024). Specifically, an equal auxin-to-cytokinin balance appears to favor initial callus induction and early morphogenic responses, while elevated cytokinin levels become

crucial during the regeneration phase, particularly for promoting embryogenic development and shoot formation at later stages of somatic embryogenesis (Asgar et al. 2023; Su et al. 2015).

Challenges in somatic embryogenesis included low rates of transformation into plantlets, a common issue in various species due to factors such as low somatic embryo quality and stress factors like ethylene and abscisic acid, which may inhibit embryo formation during the transition from the globular or torpedo stage to the cotyledon stage. This process results in a loss of totipotency efficiency in somatic embryogenesis, reducing its capacity to transform into a plant (Ochatt and Revilla 2016). Genetic and epigenetic changes under stress also contribute to these problems (Garcia et al. 2019).

The main goal in micropropagation studies is to obtain plants with the same genetic structure as the parent plant. Otherwise, undesirable differences called somaclonal variations may occur in micropropagation processes. Therefore, regeneration systems need to be evaluated in terms of genetic stability. In many previous studies, different PCR-based molecular marker techniques have been used to determine the genetic stability and somaclonal variation of different regenerated plants (Uma et al. 2021; Natarajan et al. 2020; Tippani et al. 2019). The SCoT technique is a marker system with superior advantages due to its simple, reproducible, and easily accessible features. Moreover, this technique has been reported to be more reproducible than RAPD and ISSR markers (Xiong et al. 2011; Collard and Mackill 2009). Due to the lack of sequence information for *Rheum ribes*, the SCoT marker was used to evaluate the regeneration systems with the highest number of plants in this study. Correspondingly, this study highlighted the effectiveness of the SCoT marker in detecting somaclonal variations, like its use in species like *Rumex nepalensis* (Bhattacharyya et al. 2017), *Calligonum polygonoides* (Faroda et al. 2025), and *Rumex hastatus* (Singh et al. 2025). It was determined that 20 primers yielded an average of 11.9 monomorphic and 4.75 polymorphic bands, indicating genetic variation. The PIC value ranged from 0.077 to 0.110, showing high genetic similarity, with similarity coefficients between micropropagation systems varying from 0.845 to 0.991. The highest similarity (0.991) was observed in plants from direct somatic embryogenesis in system 6 (MS + 3.0 mg/l BAP + 1.0 mg/l NAA). This supports earlier findings by Peschke and Phillips (1992) that direct culture from meristems yields genetically stable plants. The study also found that genetic stability was lower in plants regenerated through indirect organogenesis and somatic embryogenesis mediated by the callus stage. This aligns with other studies showing increased polymorphism in callus-derived plants (Rai 2023; Dilkalal et al. 2021; Manokari et al. 2021; Khor et al. 2020; Sadhu et al. 2020),

as callus formation is exposed to somaclonal variation (Adly et al. 2023). Indirect regeneration systems take longer and are more susceptible to genetic instability (Miguel and Marum 2011). The study confirmed that direct regeneration methods, like micropropagation from shoot tips, are more genetically stable than callus-based systems (Grzegorzczak-Karolak et al. 2021). Additionally, somatic embryogenesis offers better genetic stability than organogenesis due to its origin from single cells, reducing chimera formation (Giri et al. 2004; Hao and Deng 2002).

Conclusion

This study demonstrated that embryos had higher germination rates than seeds, likely due to the absence of inhibitory compounds in seed tissues. Optimal seed germination was achieved using ½ MS medium with 1.75 mg/L GA₃ and 1.0 mg/L IBA. Tissue browning was effectively controlled by adding 0.7 mg/L PVP and applying a 14-day dark pretreatment. A reproducible micropropagation protocol was developed for *Rheum ribes* using embryo axis and hypocotyl explants on MS medium with 3.0 mg/L BAP, 0.2 mg/L NAA, and 1.0 mg/L GA₃, yielding the highest regeneration (1.15 plantlets/explant). SCoT marker analysis revealed that direct regeneration yielded higher genetic stability compared to somatic embryogenesis or organogenesis. This protocol is suitable for large-scale propagation and the conservation of germplasm. As a future direction, we recommend testing optimized media enriched with endogenous hormone precursors and epigenetic modulators to further enhance regeneration efficiency.

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Author contributions M. Aydin and M. Aldaif conceived of and designed the study. M. Aldaif and S. Ucar performed the experiments. E. İlhan designed the primers and provided technical support. S. Ucar, E. Yigider and A. G. Kasapoglu conducted the molecular analyses and interpreted the results. M. Aydin performed in statistical analysis and visualization of data. M. Aldaif and E. Yigider drafted the manuscript. All the authors have read and approved the final manuscript.

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Data availability All data generated or analyzed during this study are included in this manuscript. The raw data will be made available on request.

Declarations

Conflict of interest The authors declare that they do not have any conflicts of interest.

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent for publication The authors have consented to publication.

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