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Micropropagation using direct and indirect organogenesis in *Artemisia maritima* L.: scanning electron microscopy of somatic embryos and genome size analysis by flow cytometry

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Abstract
In this study, an efficient, reproducible, and genetically stable regeneration protocol has been developed in *Artemisia maritima* L. The experiments were conducted for callus induction, plant regeneration, and somatic embryogenesis using stem and leaf of *A. maritima* as explants. The optimal callus induction (81.3%) was observed on 2.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 1.5 mg L^{-1} 6-benzylaminopurine (BAP). The shoot regeneration was observed on different concentrations of BAP, α-naphthaleneacetic acid (NAA), and thidiazuron (TDZ), using nodal segments and microshoot tips as explants. The microshoot tips were more responsive compared to nodal segments with the highest induction frequency (90.33%) obtained on 1.5 mg L^{-1} BAP. Maximum root induction frequency (74.36%) was obtained on 1.5 mg L^{-1} NAA. The somatic embryogenesis was induced on Murashige and Skoog (MS) medium amended with TDZ and indole-3-butyric acid (IBA) with maximum embryogenic induction frequency on 1.0 mg L^{-1} TDZ and 2.5 mg L^{-1} IBA. The somatic embryos developed into globular, heart-shaped, and bipolar plantlet stages on BAP and NAA as revealed through scanning electronic microscopy (SEM) and histological studies. The fully developed plants were acclimatized (75% survival rate) and transferred to natural photoperiod conditions. The DNA content and genetic stability of direct regenerated and somatic embryo–derived plants were analyzed by flow cytometry. The 2C DNA content of *in vivo* plants, direct regenerated, and somatic embryo–derived plants was 14.89, 14.61, and 14.37 pg, respectively. The genetic stability was maintained in *in vitro* cultures in comparison to field-grown plants of *A. maritima*. This study for the first time tried to formulate regeneration protocol *via* direct and indirect organogenesis and somatic embryogenesis for *A. maritima*. This paper was also the first report for comparing the 2C DNA content of *A. maritima* grown *in vivo* to *in vitro* cultured plants.

Keywords *Artemisia maritima* · DNA content · Flow cytometry · Scanning electronic microscopy · Somatic embryo

Introduction

The genus *Artemisia* (family Asteraceae) is a source of numerous phytochemical compounds (Pandey and Singh 2017; Bisht *et al*. 2021). Some species of the genus are vulnerable due to restricted distribution, numerous anthropogenic activities, and non-judicious collection to meet the increasing demands of various pharmaceutical industries (Sainz *et al.* 2017). *Artemisia maritima* L., one such species of this genus, has restricted distribution and is found in only some areas of North Western India including Kashmir, Kurram, Kishtwar, and Gurez (Parihar *et al.* 2011; Bhagat and Singh 1989; Hooker 1882). The plant is medicinally important and contains an array of phytochemicals. Artemisinin, an important antimalarial drug, has also been reported to be present in *A. maritima* (Singh *et al*. 2021). This plant possesses antihelminthic (Irum *et al*. 2015), pesticidal (Walia *et al*. 2019), anticancer (Qadir *et al*. 2019), anticytotoxicity (Qadir *et al*. 2019), antibacterial properties (Stappen *et al*. 2014), and antiplasmodial activities (Ene *et al*. 2009). According to ENVIS (2003) center on medicinal plants, with reference to Jammu and Kashmir (India), *A. maritima* is categorized as vulnerable. The populations of *A. maritima* are dwindling due to various anthropogenic activities although

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certain intrinsic factors also contribute to its dwindling populations (Bharti *et al*. 2019). So, these threats and causes advocate for *in vitro* germplasm conservation of this medicinally important species.

In vitro propagation is a powerful tool for germplasm conservation and mass multiplication (Cardoso and Da Silva 2013; Nabi *et al*. 2021; Nazir *et al*. 2021). This technique has been used for regeneration in many medicinal and aromatic plants, including several species of *Artemisia* (Ali *et al*. 2017; Al-Khayri 2018; Deepa and Thomas 2020). Micropropagation is a technique utilized for rapid multiplication, production of disease-free, uniform, and genetically stable progenies, and production of plant secondary metabolites (Zadoks 2013; Koul *et al*. 2017; Adhikary *et al*. 2021). It has various benefits over the conventional ways of propagation, including independent of season, small space requirement, and high-quality production (Kulus 2015).

Plant regeneration *via* callus formation often gives rise to somaclonal variations, which is a great challenge for the production of true-to-type plants. The somaclonal variants may arise due to epigenetic changes or permanent genetic changes (Ali *et al*. 2017; Ghosh *et al*. 2021). Variations in plants can be analyzed by various molecular markers such as amplified fragment length polymorphism (AFLP), inter-simple sequence repeats (ISSR), random amplification of polymorphic DNA (RAPD), start codon targeted (ScoT) polymorphism, and multiomics approaches (Butiuc-Keul *et al.* 2016). These methods are quite expensive and laborious and require various primers. The determination of DNA content by flow cytometry (FCM) requires a lesser amount of biological sample, is inexpensive, and can be achieved at the *in vitro* multiplication stage (Ochatt 2008; Ochatt *et al*. 2013; Miler *et al*. 2020). Since there are no reports available in the literature on micropropagation, somatic embryogenesis, and genome size analysis in *A. maritima*, this study aimed at designing an efficient reproducible micropropagation protocol for *A. maritima* in order to conserve its germplasm and development of true-to-types.

Materials and Methods

Callogenesis and Direct Plant Regeneration *A. maritima* L. plants were collected from Gurez, Bandipora, Jammu and Kashmir, India (34.38° N, 74.43° E), 2393 m above sea level (Supplementary File 1), identified under voucher no. 3146-(KASH), and established at KUBG (Kashmir University Botanical Garden, Srinagar, India). The explants (leaf and stem) were collected in a beaker containing water, washed under running tap water for about 30 min, surface sterilized by rinsing with labolene (Himedia, Maharashtra, India) for 10 min, and thoroughly washed under running tap water for the next 20 min. For about 8 min, under a laminar air flow hood, the explants were subjected to surface sterilization with 2% sodium hypochlorite (v/v) (Himedia), and washed (3 times) with autoclaved double distilled water. The explant was cut to make an appropriate size (5 to 15 mm) by trimming the dead tissue and was placed on a sterile Murashige and Skoog (MS; Murashige and Skoog 1962) medium containing 3% sucrose (w/v) (Himedia) and solidified with 0.8% agar (w/v) (Himedia). The MS medium was fortified with different plant growth regulators (PGRs) (purchased from Sigma-Aldrich, St. Louis, MO) as mentioned in Table 1. These cultures were kept at 25 ± 2 °C with 16 h of photoperiod and subcultured after every 2 wk. The treatments were performed with three replicates (1 replicate represents 3 callus pieces per test tube).

Somatic Embryogenesis and Indirect Shoot Regeneration Somatic embryogenesis was induced after successful inoculation of the green callus obtained from stem explant on MS medium supplemented with 0.5 mg L^{-1} thidiazuron (TDZ), 1.0 and 1.5 mg L^{-1} indole-3-butyric acid (IBA), and 0.5 mg L^{-1} α-naphthaleneacetic acid (NAA). Indirect shoots were observed after 2 wk of callus subculture on MS medium supplemented with different concentrations of 0.5 to 2.0 mg L^{-1} NAA and 0.5 to 2.0 mg L^{-1} IBA and their cumulative concentrations. The temperature of 25 ± 2 °C and 55 to 70% relative humidity were sustained for the cultures. All the experiments were repeated at least thrice with 12 replicates for each experiment (1 replicate represents 3 callus pieces per test tube).

Table 1. Concentration of different plant growth regulators (PGRs) fortified with Murashige and Skoog medium (*2,4-D*, 2,4-dichlorophenoxyacetic acid; *BAP*, 6-benzylaminopurine; *IAA*, indole-3-acetic acid; *KN*, kinetin; *NAA*, α-naphthaleneacetic acid; *TDZ*, thidiazuron)

PGRs used	Concentration (mg L^{-1})						
2,4-D	0.5	1.0	1.5	2.0	2.5		
BAP	0.5	1.0	1.5	2.0	2.5	3.0	3.5
NAA	0.5	1.0	1.5	2.0	2.5		
IAA	0.5	1.0	1.5	2.0	2.5		
KN	0.5	1.0	1.5	2.0	2.5		
TDZ	0.5	1.0	1.5				

Hardening and Acclimatization After washing, plants with roots were transferred to jiffy pots filled with vermiculite and maintained under 28 ± 3 °C with a relative humidity of 75 to 80%. Meanwhile, these were transferred to plastic pots containing soil, sand, and manure in the ratio of 1:1:1 for acclimatization. After 2 wk, the acclimatized plants were relocated to a greenhouse under controlled photoperiod parameters.

SEM Analysis Embryogeneic callus was fixed for 24 h at 4 °C in 2.0% glutaraldehyde, 2.0% formaldehyde, at pH 6.8 followed by washing with a buffer and fixed with 1% osmium tetraoxide, and dehydrating in ethanol graded series and finally coating with gold palladium. Lastly, samples were then photographed in EVO 18 (Carl Zeiss, Bangalore, India) scanning electron microscope functioning at 15 to 25 kV.

Histology The organogenic callus was fixed in FAA (formalin, glacial acetic acid, 70% ethanol (5:5:90), followed by dehydration in graded ethanol series and implanted in paraffin (Johansen 1940). Sections of about 8 µm were cut with the rotatory microtome (Sigma-Aldrich, St. Louis, MO). The sections were dewaxed and stained with 5% hematoxylin and 2% eosin and finally mounted on glass slides. These sections were then visualized and microphotographed (Carl Zeiss).

Flow Cytometry In this study, genome size analysis was performed for field-grown plants, plants regenerated *via* direct organogenesis, and somatic embryo–derived plantlets. For this purpose, three randomly selected plants were analyzed from each experimental setup of *in vitro* grown *Artemisia* (plants regenerated *via* direct organogenesis, and somatic embryogenesis), and from *in vivo* grown control group. Plant material of *Artemisia maritima* nearly 1.0 cm^2 and reference standard [*Pisum sativum* (2C DNA = 9.56 pg)] were meshed in 0.5 mL Otto buffer (0.3% citric acid monohydrate, 0.05% NP-40), 50 µg mL^{-1} propidium iodide, and 100 µg mL^{-1} Rnase (Sigma-Aldrich). The genomic samples prepared were sieved through a 100 µm mesh sieve, before being examined through (CFM) BD FACS Calibur (BD Biosciences, San Jose, CA) flow cytometer (Dolezel *et al*. 2007). Nuclear DNA content (2C) of *A. maritima* was calculated using the below formula:

$$\text{2C DNA content of } A.\ maritima \text{ L.} = 9.56 \times \frac{\text{Mean position of G0/G1 } peak\ of\ A.maritmaL.}{\text{Mean position of G0/G1 peak of } Pisum\ sativum}$$

Statistical Analysis All the treatments were performed in replicates ($n = 3$), and the presented data was expressed as mean ± standard deviations (SD). Significant differences between the mean values were assessed by one-way ANOVA followed by *post hoc* Duncan's multiple range tests (DMRT) at $p \leq 0.05$, using SPSS software (version 22, IBM Armonk, New York).

Results

Callus Induction and Proliferation Callus induction was witnessed in stem and leaf explants inoculated on MS media amended with varying concentrations of auxins [2,4-dichlorophenoxyacetic acid (2,4-D), indole-3-acetic acid (IAA), IBA, and NAA] and cytokinins [6-benzylaminopurine (BAP) and kinetin (KN)]. The best callus induction was observed on 2.5 mg L^{-1} 2,4-D in combination with 1.5 mg L^{-1} BAP. The percent response of stem varied from 38.65% (0.5 mg L^{-1} 2,4-D and 0.5 mg L^{-1} BAP) to 81.32% (2.5 mg L^{-1} 2,4-D and 1.5 mg L^{-1} BAP) (Fig. 1*a* to *d*). Similarly, in the case of leaf explant, the percent response varied from 30.64% (0.5 mg L^{-1} IAA) to 81.26% (1.5 mg L^{-1} 2,4-D and 1.0 mg L^{-1} BAP) (Fig. 1*e* to *h*) (Table 2). The color and texture of callus varied among different growth regulators (Fig. 1*a* to *h*). After 4 wk, callus was subcultured on different concentrations of BAP, NAA, and KN (0.5 to 3.5 mg L^{-1}). Among different concentrations, the best proliferation was obtained on MS media fortified with NAA and KN (each 1.0 mg L^{-1}) with a fresh weight of 4.02 ± 0.38 g from stem-originated callus and 3.96 ± 0.35 g from leaf-originated callus, which were significantly different at $p \leq 0.05$ according to DMRT (Table 3). Therefore, stem-originated callus proliferated better in comparison to leaf-originated callus. The maximum callus induction frequency and proliferation thus resulted from the cumulative effect of growth hormones.

Shoot Regeneration The nodal and microshoot tips were aseptically inoculated on MS media amended with 0.5 to 2.5 mg L^{-1} BAP, 0.5 to 1.5 mg L^{-1} NAA, and 0.5 to 1.5 mg L^{-1} TDZ. The best direct shoot regeneration was obtained on 1.0 mg L^{-1} BAP in combination with 0.5 mg L^{-1} NAA with 85.25% induction frequency on nodal explant and 90.26% induction frequency on microshoot tips (Table 4). The mean number of shoots per node varied among different concentrations of growth hormones. The maximum number of shoots was noticed on 1.5 mg L^{-1} BAP in combination with 1.0 mg L^{-1} NAA. The mean number of shoots was 85.24 ± 3.67 on nodal segments and 88.34 ± 3.82 on microshoot tips after 4 wk of inoculation (Table 4) (Fig. 2*a* to *d*).

Indirect Shoot Regeneration After 3 wk of inoculation, a well-established callus resulted in indirect shoot regeneration on MS media amended with 0.5 mg L^{-1} TDZ, 0.5 mg L^{-1} NAA, and 1.0 to 1.5 mg L^{-1} IBA (Fig. 3*a* to *d*). The maximum *in vitro* indirect shoot regeneration induction was obtained on 0.5 mg L^{-1} TDZ in combination with 1.5 mg L^{-1} IBA with 80.36% induction frequency and 28.71 ± 0.51 mean number of shoots (Table 5).

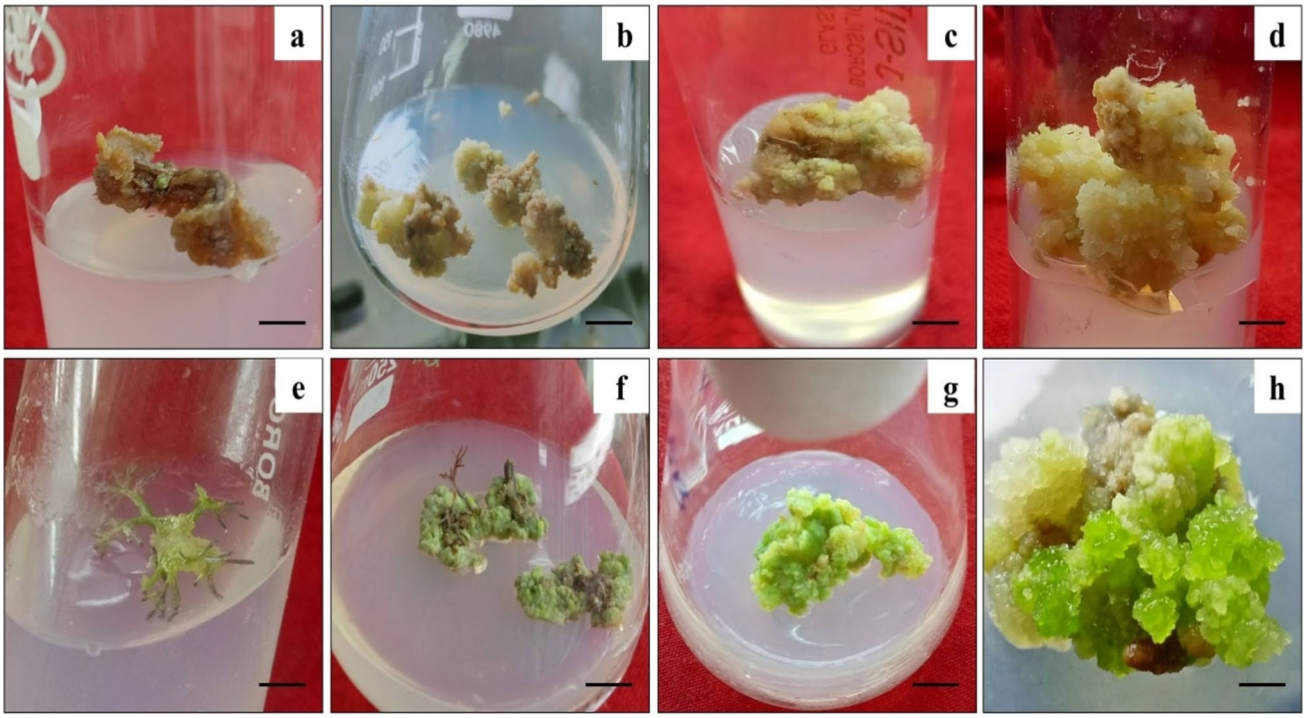

Figure 1. Callus induction and proliferation in stem and leaf explants of *Artemisia maritima* L. (*a*) Brown callus (stem explant) obtained in Murashige and Skoog (MS) medium amended with 2.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D). (*b*) Brown callus from leaf explant (MS medium amended with 2.0 mg L^{-1} 2,4-D). (*c*) White yellowish callus from stem (MS medium added with 2.0 mg L^{-1} α-naphthaleneacetic acid (NAA)). (*d*) Callus proliferation. (*e*) Green callus from leaf explant (MS medium amended with 1.5 mg L^{-1} 2,4-D and 1.0 mg L^{-1} 6-benzylaminopurine (BAP)). (*f*) green callus from stem explant showing regenerated leaves (MS medium amended with 2.5 mg L^{-1} 2,4-D and 1.5 mg L^{-1} BAP). (*g–h*) callus proliferation (MS medium amended with 1.0 mg L^{-1} NAA and 1.0 mg L^{-1} kinetin (KN)) (*scale bars a–h*: 0.5 cm).

Rooting The *in vitro* cultured shoots were transferred to MS media amended with varying concentrations of 0.5 to 1.5 mg L^{-1} NAA and 0.5 to 2.0 mg L^{-1} IBA. The higher root formation frequency was observed on 1.5 mg L^{-1} NAA (74.36%) with 14.82 ± 0.70 mean number of roots (Table 6). However, no root formation was observed on IAA which instead resulted in callus formation below the shoot.

Somatic Embryogenesis The callus obtained from 2,4-D (brown) and NAA (light yellowish white) failed to form the somatic embryos (SEs) while the callus obtained on BAP and KN resulted in the SE formation (Fig. 4*a* to *f*) when placed on MS media amended with 0.5 to 1.0 mg L^{-1} TDZ and 1.5, 2.0, and 2.5 mg L^{-1} IBA (Table 7). The germination of SEs was observed on MS media amended with 1.0 to 3.5 mg L^{-1} BAP and 0.5 to 2.0 mg L^{-1} NAA (Fig. 4*a* to *h*) (Table 8).

Acclimatization of Plantlets The plantlets were successfully acclimatized under greenhouse conditions (Fig. 5) following Chandra *et al*. (2010). The plants were healthy and true-to-type showing a 75% of survival rate. No phenotypic variation was noticed between *in vivo* grown and regenerated plants.

Histology Histological examination of differentiated shoot from callus tissue showed various developmental stages of apical meristem having dome-shaped apical meristem with early leaf primordia (Supplementary File 2). A shoot apex partially covered with two leaf primordia was witnessed at advanced stages. A zone of compact meristematic tissue was detected close to the apex. As shown in Supplementary File 2, embryogenic tissues were observed, which were composed of meristematic cells with dense cytoplasm and prominent nuclei. Subsequently, embryonic cells continued to differentiate, and globular embryos gradually formed after 8 wk (Supplementary File 2).

SEM Analysis SEM observations revealed the external surface of the embryogenic callus with irregular and frequent protuberances. The emergence of globular SEs from the surface of the embryogenic callus was confirmed by SEM micrographs. The globular SEs then developed through later stages (Supplementary File 2). After 3 wk of callus subculture, the adventitious shoot primordia originated.

Genetic Stability and Genome Size Analysis The FCM was used to quantify 2C DNA content in order to assess

Table 2. Callus induction frequency (%), callus texture, and color in stem and leaf explants of *Artemisia maritima* L. grown in Murashige and Skoog medium amended with different concentrations of plant growth regulators (PGRs) with three replicates for each treatment (1 replicate = 3 callus pieces per test tube) (*2,4-D*, 2,4-dichlorophenoxyacetic acid; *BAP*, 6-benzylaminopurine; *IAA*, indole-3-acetic acid; *KN*, kinetin; *NAA*, α-naphthaleneacetic acid)

PGRs (mg L^{-1})					Stem explant		Leaf explant	
2,4-D	BAP	IAA	NAA	KN	Callus induction (%)	Callus texture and color	Callus induction (%)	Callus texture and color
0.5					42.30 ± 2.11[z]e[y]	Brown and friable	38.23 ± 2.14e	Brown and friable
1					48.61 ± 1.82d	Brown and friable	41.62 ± 2.37d	Brown and friable
1.5					51.22 ± 2.31c	Brown and friable	43.67 ± 4.40d	Brown and friable
2					66.31 ± 1.93b	Brown and friable	56.17 ± 3.63c	Brown and friable
2.5					72.30 ± 2.62a	Brown and friable	52.33 ± 3.42c	Brown and friable
	0.5				41.64 ± 1.92e	Green and hard	30.64 ± 1.37e	Green and friable
	1				44.86 ± 3.11d	Green and hard	33.42 ± 1.88e	Green and friable
	1.5				49.32 ± 1.56d	Green and hard	43.62 ± 2.36d	Green and friable
	2				52.31 ± 2.34c	Green and hard	46.85 ± 3.19d	Green and friable
	2.5				59.32 ± 2.53c	Green and hard	51.24 ± 3.65c	Green and friable
	3				63.62 ± 2.42b	Green and hard	58.32 ± 3.82c	Green and friable
	3.5				71.44 ± 3.61a	Green and hard	54.31 ± 3.28c	Green and friable
		0.5		0.5	40.82 ± 1.16d	Green and hard	36.82 ± 3.16e	Green and hard
		0.5		1	48.62 ± 2.44c	Green and hard	43.31 ± 3.87d	Green and hard
		1		2	53.61 ± 2.32c	Green and hard	53.84 ± 3.13c	Green and hard
		1.5		2.5	71.52 ± 3.27a	Green and hard	59.35 ± 3.34c	Green and hard
		2		0.5	70.62 ± 3.72a	Green and hard	61.32 ± 3.87b	Green and hard
		2.5		0.5	64.81 ± 3.80b	Green and hard	63.31 ± 3.22b	Green and hard
			0.5		40.82 ± 1.54e	Light yellowish white and friable	30.25 ± 3.16e	Light yellowish white and friable
			1		46.13 ± 2.12d	Light yellowish white and friable	33.61 ± 1.82e	Light yellowish white and friable
			1.5		51.61 ± 3.26d	Light yellowish white and friable	39.20 ± 1.95d	Light yellowish white and friable
			2		62.82 ± 3.47b	Light yellowish white and friable	43.68 ± 2.81d	Light yellowish white and friable
			2.5		55.66 ± 3.10c	Light yellowish white and friable	51.66 ± 2.90c	Light yellowish white and friable
0.5	0.5				38.65 ± 3.65e	Green and hard	41.23 ± 3.22d	Green and friable
1	0.5				42.30 ± 3.26d	Green and hard	52.65 ± 2.47c	Green and friable
1.5	1				70.27 ± 3.62a	Green and hard	81.26 ± 2.62a	Green and friable
2	1				71.22 ± 4.14a	Green and hard	73.23 ± 2.91a	Green and friable
2.5	1.5				81.32 ± 4.53a	Green and hard	71.20 ± 3.43b	Green and friable
3	1.5				69.33 ± 3.87b	Green and hard	70.24 ± 3.77b	Green and friable

[z]Data was recorded 4 wk after inoculation and represents mean ± SD ($n = 3$)

[y]Data within a *column* followed by dissimilar *small case letters* are significantly different at $p \leq 0.05$ by Duncan's multiple range test

the genetic stability of field-grown and *in vitro* regenerated plantlets of *A. maritima* L. For FCM, leaf samples were chopped to obtain nuclear suspensions of field-grown (*in vivo*) and *in vitro* regenerated plants (direct organogenesis and somatic embryo–derived). The histograms obtained from FCM analysis confirmed the DNA content (Supplementary File 3). The field-grown plants of *A. maritima* L. were estimated to contain 14.89 pg of 2C DNA. The 2C DNA content of direct organogenesis derived (14.61 pg) and somatic embryo–derived (14.37 pg) regenerants depicted similarity in their genome size with the field-grown plants (Table 9). Hence, in the present protocol, the obtained regenerants maintained their genetic stability and showed no change in genome size.

Table 3. Callus proliferation in stem and leaf explants of *Artemisia maritima* L. in terms of fresh weight (g), after 4 wk of inoculation, under different plant growth regulators (PGRs) with three replicates for each treatment (1 replicate = 3 callus pieces per test tube) (*BAP*, 6-benzylaminopurine; *NAA*, α-naphthaleneacetic acid; *KN*, kinetin)

PGRs (mg L^{-1})			Callus fresh weight (g)	
BAP	NAA	KN	**Stem explant**	**Leaf explant**
0.5			0.968 ± 0.14[z]e[y]	0.583 ± 0.11e
1			1.672 ± 0.34d	0.661 ± 0.09e
1.5			1.711 ± 0.15d	0.674 ± 0.10e
2			1.712 ± 0.14d	0.744 ± 0.13d
2.5			1.782 ± 0.28d	0.890 ± 0.19d
3			2.234 ± 0.18c	0.962 ± 0.17c
3.5			3.868 ± 0.23b	1.016 ± 0.19c
	0.5	0.5	2.112 ± 0.35c	1.893 ± 0.44b
	1	1	4.023 ± 0.38a	3.962 ± 0.35a
	1.5	1	3.861 ± 0.27b	3.322 ± 0.29a
	2	1.5	3.289 ± 0.11b	2.819 ± 0.23b
0.5		0.5	1.689 ± 0.33d	1.425 ± 0.26c
0.5		1	1.928 ± 0.41d	1.822 ± 0.38b
1		1	2.315 ± 0.53c	2.156 ± 0.45b

[z]Data was recorded 4 wk after inoculation and represents mean ± SD ($n = 3$)

[y]Data within a *column* followed by dissimilar *small case letters* are significantly different at $p \leq 0.05$ by Duncan's multiple range test

Discussion

In this study, a novel and well-established protocol was developed for callus induction, somatic embryogenesis, and *in vitro* regeneration of *A. maritima* L. The callus induction response from leaf and stem explants was observed on almost all concentrations of different growth hormones like 2,4-D, NAA, and BAP. Similar results were also found in *Artemisia annua* in which callogenesis was observed on all combinations of BAP, NAA, and 2,4-D (Zayova *et al*. 2020). In this study, the maximum callus induction frequency was observed on 2.5 mg L^{-1} 2,4-D and minimum frequency on 1.5 mg L^{-1} BAP. Similar observations were noted in *A. annua* (Zayova *et al*. 2020). Callus has a variable appearance in texture and shape (Mohajer *et al*. 2012; Sikdar *et al*. 2012). Also, in the present study, the color, texture, and embryogenic potential of callus varied with the use of different growth hormones (Table 2; Fig. 1*a* to *h*). In the present study, brown, friable, non-embryogenic callus was observed on 2,4-D, while green, hard, and embryogenic callus was seen in plants cultured on BAP and light yellowish white friable and non-embryogenic callus was observed in NAA, on both leaf and stem explants. Aslam *et al*. (2006) noted similar observations in *Artemisia scoparia*. The brown and

Table 4. Direct shoot regeneration induction frequency (%) using nodal and microshoot tips as explants in *Artemisia maritima* L., after 3 wk of inoculation, using different plant growth regulators (PGRs) with three replicates for each treatment (1 replicate = 3 callus pieces per test tube) (*BAP*, 6-benzylaminopurine; *NAA*, α-naphthaleneacetic acid; *TDZ*, thidiazuron)

PGRs (mg L^{-1})			Shoot induction (%)		Mean number of shoots per explant	
BAP	NAA	TDZ	**Nodal explant**	**Microshoot tips explant**	**Nodal explant**	**Microshoot tips explant**
0.5			41.22 ± 2.12[z]d[y]	38.62 ± 1.85e	3.32 ± 0.31e	5.84 ± 0.53e
1			62.41 ± 3.85c	51.23 ± 2.36d	7.81 ± 0.63e	8.81 ± 0.42e
1.5			80.23 ± 4.22a	90.33 ± 4.19a	28.31 ± 1.62c	19.20 ± 1.16d
2			76.24 ± 3.58a	79.36 ± 3.66a	26.23 ± 0.83c	20.61 ± 0.72d
2.5			71.28 ± 2.86b	69.85 ± 3.21c	36.11 ± 1.87c	43.36 ± 2.37c
	0.5		38.26 ± 1.59e	39.61 ± 2.51e	7.80 ± 0.42e	10.55 ± 0.63d
	1		43.86 ± 1.86d	47.25 ± 1.92d	11.62 ± 0.51d	15.82 ± 0.84d
	1.5		58.35 ± 3.11c	61.26 ± 3.26c	20.33 ± 1.16c	26.36 ± 1.57d
1	0.5		85.25 ± 4.12a	90.26 ± 3.85a	64.22 ± 3.22b	61.23 ± 3.15b
1.5	1		73.26 ± 3.28b	76.38 ± 2.55b	85.24 ± 3.67a	88.34 ± 3.82a
2	1.5		63.25 ± 3.32c	68.35 ± 2.88c	55.47 ± 1.92b	61.22 ± 2.16b
		0.5	32.25 ± 2.26e	34.26 ± 2.15e	8.22 ± 0.67e	15.88 ± 0.77d
		1	41.02 ± 1.89d	42.36 ± 1.29d	17.66 ± 0.82d	22.83 ± 0.73d
		1.5	58.26 ± 2.33c	61.28 ± 2.36c	21.28 ± 0.96c	33.67 ± 1.12c

[z]Data was recorded 3 wk after inoculation and represents mean ± SD ($n = 3$)

[y]Data within a *column* followed by dissimilar *small case letters* are significantly different at $p \leq 0.05$ by Duncan's multiple range test

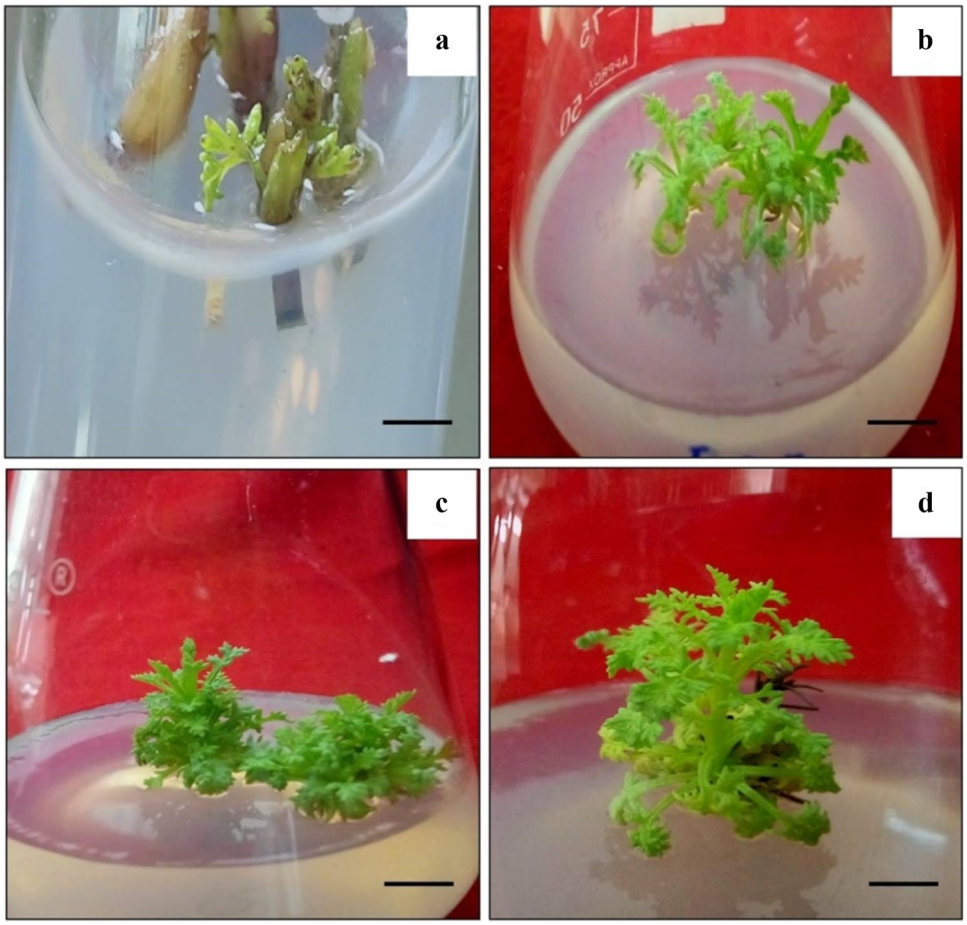

Figure 2. Direct shoot regeneration from nodal and microshoot tip explants of *Artemisia maritima* L. (*a–b*) Nodal shoot regeneration in Murashige and Skoog (MS) medium amended with 1.0 mg L^{-1} 6-benzylaminopurine (BAP) and 0.5 mg L^{-1} α-naphthaleneacetic acid (NAA). (*c–d*) Regeneration from microshoot tip in 1.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA added MS medium (*scale bars a–d*: 0.5 cm).

yellowish white callus failed to grow after two subcultures in comparison to the green callus obtained from stem explant while the green callus obtained from the leaf explant also showed browning after two subcultures. The fresh weight of callus after 4 wk of subculturing on different media also varied. The shoot regeneration from callus was observed on TDZ, NAA, and IBA. Similar results were observed by Boo *et al*. (2015) in *Aster scaber* in which shoot regeneration from callus was observed on 5 to 25 µM NAA and 0.05 to 25 µM BAP.

Direct shoot regeneration was observed on different concentrations of BAP, TDZ, and NAA using nodal and microshoot tips as explants. The microshoot tips were more responsive as compared to nodal segments. The maximum shoot induction was detected on 1.0 mg L^{-1} BAP in combination with 0.5 mg L^{-1} NAA. In *Artemisia spicegera*, *in vitro* shoots were observed on 0.5 mg L^{-1} NAA and 0.5 mg L^{-1} BAP (Ghorbani *et al*. 2021). The present study's results resembled the observations of Lualon *et al*. (2008) in which shoot regeneration was also observed on BAP that was found to be maximum on 0.1 mg L^{-1} TDZ in combination with 0.05 mg L^{-1} NAA and 1.0 mg L^{-1} BAP. Similar observations were also found by Dangash *et al*. (2015) on media amended with 1.5 mg L^{-1} BAP and 0.05 mg L^{-1} NAA. In *Alocosia longiloba*, the maximum number of shoots was observed on 3.0 mg L^{-1} BAP (Abdulhafiz *et al*. 2020).

Rooting was induced from *in vitro* regenerated plants using IBA and NAA. The highest root induction frequency was observed on 1.5 mg L^{-1} NAA. Ghorbani *et al*. (2021) also noted the rooting on 1.0 mg L^{-1} NAA and 1.0 mg L^{-1} IBA. NAA, IBA, and IAA were found to be potent root inducers in *Artemisia nilagirica* var. nilagirica (Shinde *et al*. 2016). IBA (2.4, 4.9, 9.8 µM) was also assessed for *in vitro* rooting in *Artemisia annua* (Wetzstein *et al*. 2018). In the present study, concentrations of 0.5 to 4.0 mg L^{-1} of IAA were used for rooting but no roots were observed on IAA. The present study's results were in similarity with Jogam *et al*. (2020) where rooting on regenerated *Artemisia vulgaris* shoots was observed on MS media augmented with 1.0 mg L^{-1} IBA.

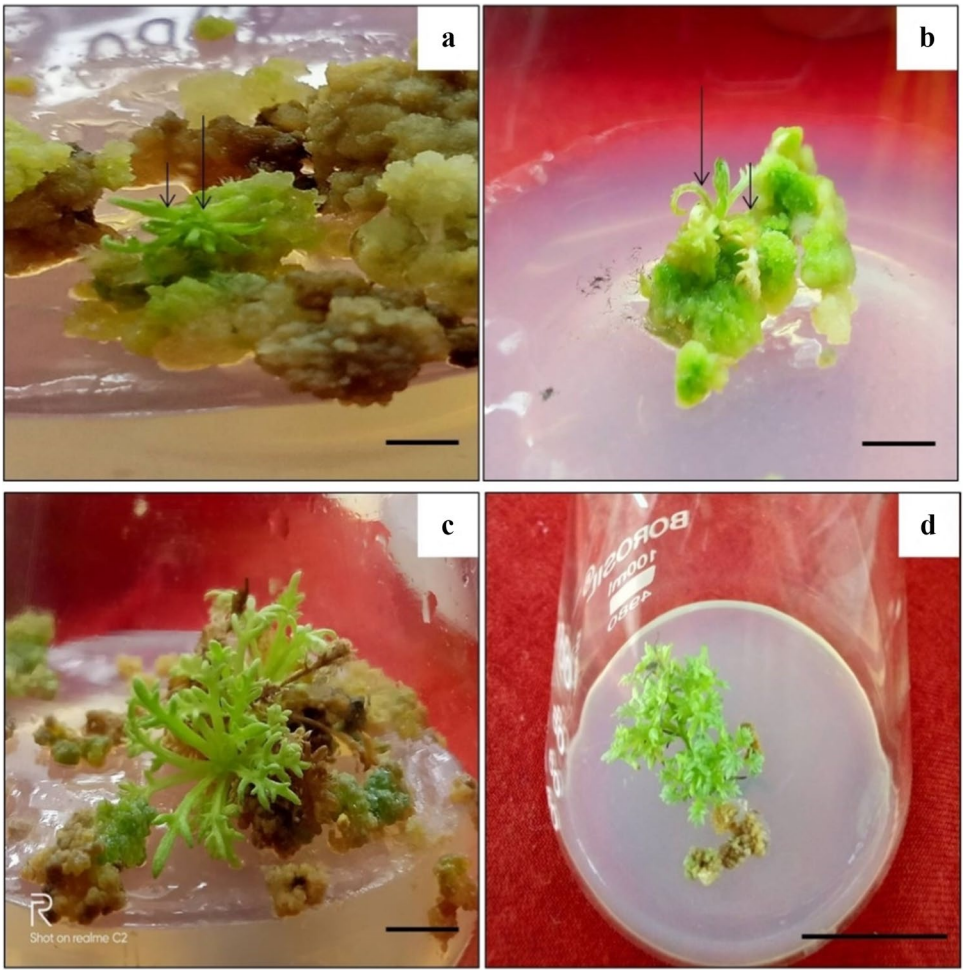

Figure 3. Indirect shoot regeneration *via* callus formation in *Artemisia maritima* L. (*a*) Early shoot bud initiation. (*b*) Little elongation of shoot. (*c*) Multiple shoot regeneration. (*d*) Fully developed plant on rooting media (*scale bars a–c*: 0.5 cm, *d*: 1 cm).

Table 5. Indirect shoot induction frequency (%) from callus (derived from 2.0 mg L^{-1} NAA and 1.5 mg L^{-1} KN; stem explant) in *Artemisia maritima* L., after 3 wk of subculturing, using different plant growth regulators (PGRs) with three replicates for each treatment (1 replicate = 3 callus pieces per test tube) (*IBA*, indole-3-butyric acid; *NAA*, α-naphthaleneacetic acid; *TDZ*, thidiazuron; *KN*, kinetin)

PGRs (mg L^{-1})			Shoot induction (%)	Mean number of shoots per callus
TDZ	NAA	IBA		
0.5		1	50.23 ± 2.36[z]b[y]	6.82 ± 0.42d
0.5		1.5	80.36 ± 1.86a	28.71 ± 0.51a
	0.5	1	45.85 ± 2.36c	23.63 ± 0.83b
	0.5	1.5	25.38 ± 2.11d	12.41 ± 1.82c

[z]Data was recorded 3 wk after subculturing and represents mean ± SD ($n = 3$)

[y]Data within a *column* followed by dissimilar *small case letters* are significantly different at $p \leq 0.05$ by Duncan's multiple range test

Somatic embryogenesis is an important application of plant tissue culture for rapid and mass propagation of plants, germplasm conservation, and genetic improvement (Guan *et al*. 2016; Zhang *et al*. 2021). The green callus obtained

Table 6. Effect of plant growth regulators (PGRs), NAA, and IBA, on rooting of *in vitro* regenerated shoots derived from direct/indirect organogenesis of *Artemisia martima* L. with three replicates for each treatment (1 replicate = 3 callus pieces per test tube) (*NAA*, α-naphthaleneacetic acid; *IBA*, indole-3-butyric acid)

PGRs (mg L^{-1})		Root induction (%)	Mean number of roots per explant
NAA	IBA		
0.5		42.68 ± 1.82[z]d[y]	5.22 ± 0.43c
1		53.68 ± 1.92b	7.51 ± 0.62b
1.5		74.36 ± 2.36a	14.82 ± 0.70a
2		61.26 ± 2.19b	13.33 ± 0.43b
	0.5	33.26 ± 1.45d	3.42 ± 0.22c
	1	46.39 ± 2.18c	6.17 ± 0.36b
	1.5	69.35 ± 3.16b	11.35 ± 0.41a
	2	53.89 ± 2.88b	9.10 ± 0.30b
0.5	1.5	48.36 ± 4.11c	8.33 ± 0.42b
1	1.5	59.68 ± 3.26b	12.21 ± 0.66a
1.5	2	52.36 ± 3.14c	11.76 ± 0.57a

[z]Data was recorded 4 wk after inoculation and represents mean ± SD ($n = 3$)

[y]Data within a *column* followed by dissimilar *small case letters* are significantly different at $p \leq 0.05$ by Duncan's multiple range test

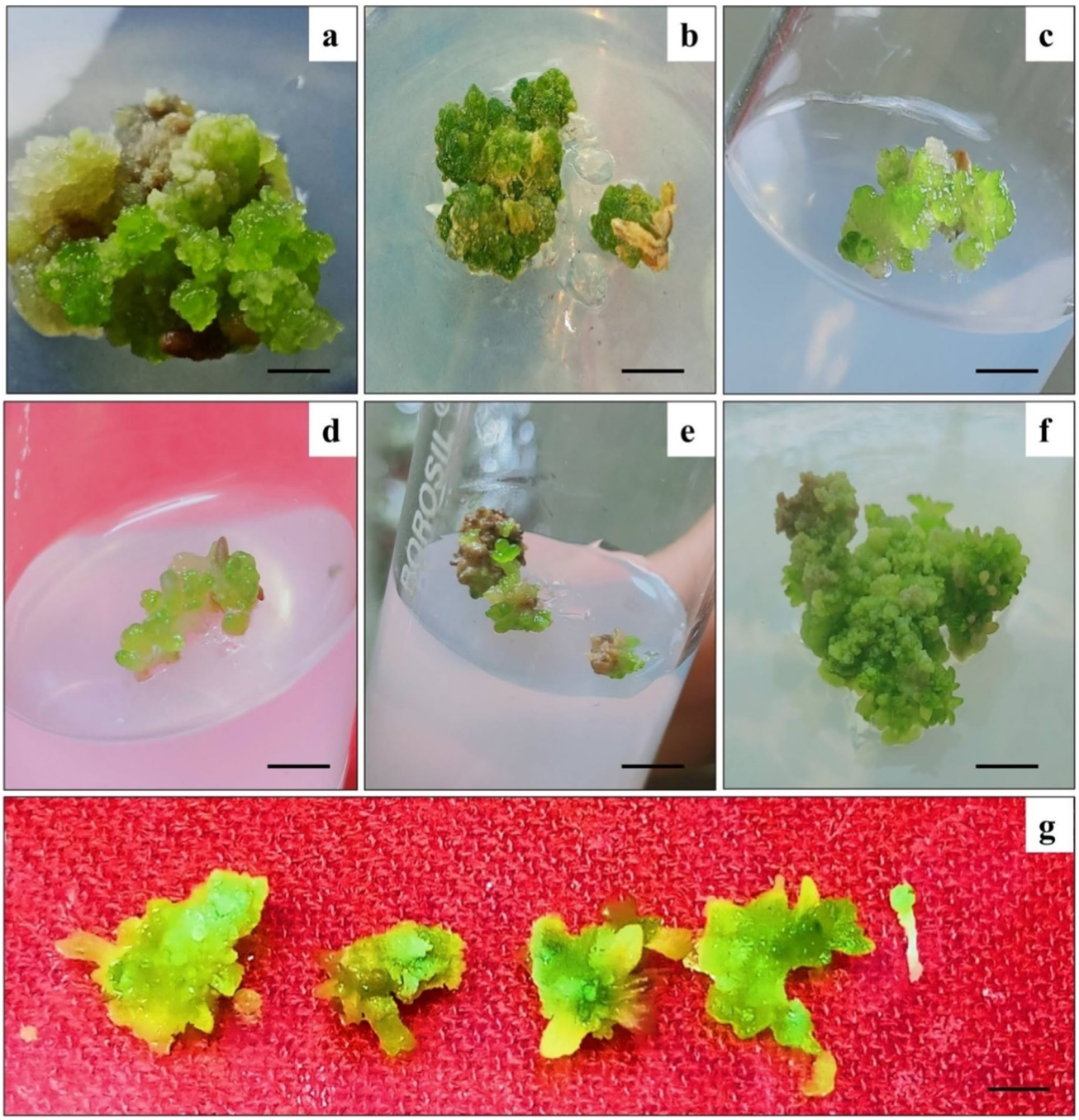

Figure 4. Various stages of somatic embyogenesis in *Artemisia maritima* L. grown in Murashige and Skoog medium. (*a*) Granular embryogenic callus. (*b–c*) Globular stage. (*d–e*) Heart-shaped stage. (*f*)Germinating embryos with emerging shoot buds (*arrows*). (*g–h*) Somatic embryos isolated from embryogenic callus in various stages, radicle and emerging shoot (*arrows*) (*scale bars a–h*: 0.5 cm).

from stem explant resulted in embryogenic callus formation whereas the brown and yellowish white callus resulted in non-embryogenic callus. Similarly, results were obtained in *Camellia oleifera* Abel (Zhang *et al*. 2021). Somatic embryogenesis is highly influenced by various parameters like the source of explant, age of culture, growth hormones, and cultural conditions (Varis *et al*. 2018; Hapsoro *et al*. 2020). In this study, the SE induction was observed on different concentrations of TDZ (0.5 and 1.0 mg L^{-1}) and BAP (1.5, 2.0, and 2.5 mg L^{-1}). The germination of SEs is a crucial step in the regeneration of a whole plant. In this study, germination of SEs was observed on 1.0 to 2.5 mg L^{-1} BAP

Table 7. Somatic embryogenesis induction frequency (%) in stem explant–derived callus (grown in 3.5 mg L^{-1} BAP) of *Artemisia maritima* L. cultured in Murashige and Skoog medium amended with plant growth regulators (PGRs) TDZ and IBA with three replicates for each treatment (1 replicate = 3 callus pieces per test tube) (*BAP*, 6-benzylaminopurine; *IBA*, indole-3-butyric acid; *TDZ*, thidiazuron)

PGRs (mg L^{-1})		Frequency of embryogenesis (%)	Mean number of somatic embryos per culture
TDZ	IBA		
0.5	1.5	25.68 ± 2.51[z]c[y]	1.93 ± 0.22c
1	2	38.56 ± 3.11b	2.93 ± 0.36b
1	2.5	46.11 ± 4.27a	4.47 ± 0.54a

[z]Data was recorded 8 wk after inoculation and represents mean ± SD ($n = 3$)

[y]Data within a *column* followed by dissimilar *small case letters* are significantly different at $p \leq 0.05$ by Duncan's multiple range test

Table 8. Somatic embryos germination frequency (%) in stem explant–derived callus (grown in 1.0 mg L^{-1} thidiazuron (TDZ) in combination with 2.5 mg L^{-1} indole-3-butyric acid (IBA)) of *Artemisia maritima* L. on Murashige and Skoog medium amended with different concentrations of the plant growth regulators (PGRs) BAP (6-benzylaminopurine) and NAA (*α-naphthaleneacetic acid)*

PGRs (mg L^{-1})		Germination (%)	Mean number of plantlets/culture
BAP	NAA		
1	0.5	32.36 ± 2.11[z]d[y]	4.26 ± 0.32d
1.5	0.5	47.61 ± 3.26c	5.26 ± 0.53c
2	1.5	68.56 ± 4.15a	7.09 ± 0.40a
2.5	1.5	56.34 ± 4.76b	6.12 ± 0.72b
3.5	2	51.21 ± 5.18b	6.19 ± 0.87b

[z]Data was recorded 4 wk after inoculation and represents mean ± SD ($n = 3$)

[y]Data within a *column* followed by dissimilar *small case letters* are significantly different at $p \leq 0.05$ by Duncan's multiple range test

and 0.5 to 2.0 mg L^{-1} NAA. In *Solanum nigrum*, the somatic embryogenic induction and germination were observed on 0.5 mg L^{-1} NAA and 1.0 to 3.0 mg L^{-1} BAP, and 0.5 mg L^{-1} BAP and 0.5 to 6.0 mg L^{-1} NAA, respectively (Sharada *et al*. 2019). In *Carica papaya*, 100% germination of SEs was also observed on 0.2 mg L^{-1} NAA and 0.2 mg L^{-1} BAP (Bukhori 2013). After culturing the SEs onto MS medium added with 0.5 mg L^{-1} BAP, the SEs developed into shoots (Ku and Chan 2013). Lema-Rumińska *et al*. (2019) induced SEs on BAP and NAA in *Echinacea purpurea*.

The histological and morphological studies also confirmed the development of SEs. In many other observations, histological, morphological, and SEM analyses were used for confirmation of SEs (Aslam *et al*. 2014; Shashi and Bhat 2021 *(Cenchrus ciliaris)*; Haradzi *et al*. 2021 (*Citrus* x *meyeri*)).

The plants originated *via* tissue culture may show genetic variation. This instability can be checked by various molecular assays, cytogenetic analysis, and biotechnological tools (Das *et al*. 2013). FCM is often used for rapid and reliable estimation of DNA content and change in ploidy levels (Bennett and Leitch 2005; Bennett and Leitch 2011). In this study's experimentation, FCM analysis of *in vivo* plant, direct regenerated plant, and somatic embryogenic plant was performed for genome size analysis and genetic stability. The peaks of 2C DNA content of *in vivo* plant, direct regenerated plant, and somatic embryo regenerated plant showed similarity and were almost similar to *in vivo* plants. Thus, the DNA contents of tissue culture–raised *A. maritima* L. plants were unaltered. The genome size analysis and ploidy identification could be investigated by FCM for both *in vivo* plants and *in vitro* regenerated plants (Sliwinska and Thiem 2007; Sliwinska 2018). Similar genome size stability has been testified in other *in vitro* grown plants like *Eucalyptus globules* (Ribeiro *et al*. 2016), *Camellia sinensis* L. (Samarina *et al*. 2019), *Coriander sativum* (Ali *et al*. 2017), and strawberry cultivars (Naing *et al*. 2019). In certain cases, *in vitro* stresses brought about genome variability as was eminent in regenerated plants of *Elaeis guineensis* (Giorgetti *et al*. 2011).

Conclusions

In conclusion, this study established a novel, efficient, and genetically stable protocol for *in vitro* regeneration of *Artemisia maritima* L. *via* both organogenesis (direct and indirect) and somatic embryogenesis. The optimal callus induction was obtained on 2.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 1.5 mg L^{-1} 6-benzylaminopurine (BAP), and shoot regeneration was observed on different concentrations of BAP, α-naphthaleneacetic acid (NAA), and thidiazuron (TDZ) using nodal segments and microshoot tips as explants. Maximum rooting was obtained on 1.5 mg L^{-1} NAA, and the maximum somatic embryogenic induction frequency was induced on MS medium amended with 1.0 mg L^{-1} TDZ and 2.5 mg L^{-1} indole-3-butyric acid (IBA). The establishment of

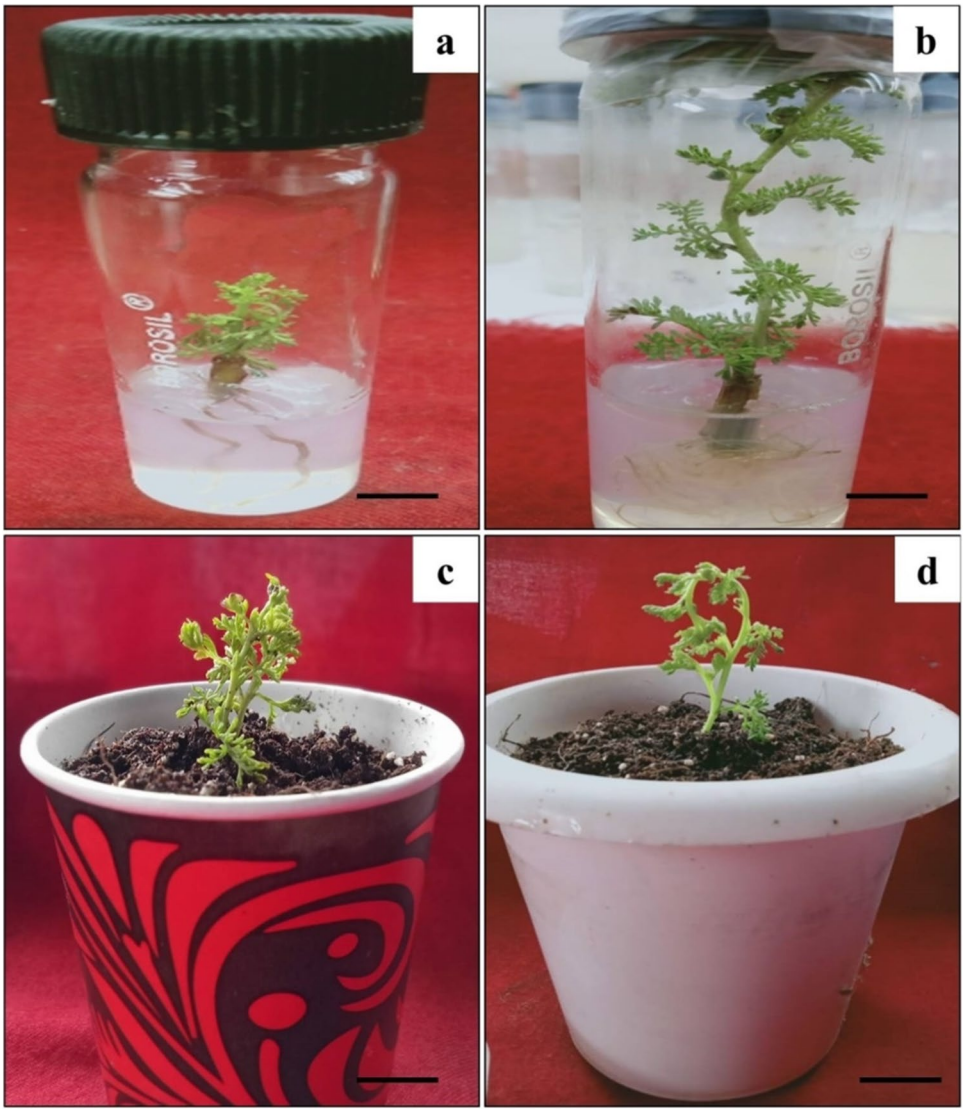

Figure 5. Rooting induction, hardening, and acclimatization in *Artemisia maritima* L. (*a–b*) *In vitro* root induction in shoots grown in Murashige and Skoog medium amended with 1.5 mg L^{-1} α-naphthaleneacetic acid, after 4 wk of culture. (*c*) Plant acclimatization in pots (*scale bars a–b*: 0.5 cm, *c*: 2.0 cm).

somatic embryos can be utilized for the development of whole plant and true-to-types as the genetic stability was confirmed by flow cytometry. The standardized protocol would be useful in germplasm conservation and the formation of high-value clonal regenerants for commercial production. The somatic embryos could be utilized for the development of synthetic seeds and grown anywhere.

Table 9. 2C DNA content [in picograms (pg)] in *in vivo* and *in vitro* derived plantlet of *Artemisia maritima* L. as detected by flow cytometry

Plantlet sources	DNA content (pg)	Coefficient of variation (%)
In vivo grown	14.89 ± 0.30[z]a[y]	8.0
Direct organogenic	14.61 ± 1.06a	6.2
Somatic embryo–derived plantlets	14.37 ± 0.74a	7.5

[z]Data represents mean ± SD ($n = 3$)

[y]Data within a *column* followed by similar *small case letters* are not significantly different at $p \leq 0.05$ by Duncan's multiple range test

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Author Contribution All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Neelofer Nabi and Peer Saffeullah. The first draft of the manuscript was written by Neelofer Nabi and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data Availability All data generated or analyzed during this study are included in this published article.

Declarations

Conflict of Interest The authors declare no competing interests.

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