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Towards *ex situ* conservation of globally rare Turkish endemic *Tripleurospermum fissurale* (Asteraceae)

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Abstract

A rapid and efficient *in vitro* micropropagation system was developed to conserve *Tripleurospermum fissurale* (Sosn.) E.Hossain (Asteraceae), a rare endemic species for Turkey. Murashige and Skoog basal medium (MS) supplemented with 4.7 µM KIN was found to be the most appropriate basal medium with a 60% germination rate. MS medium supplemented with 4.9 µM 2iP and 0.5 µM IBA gave the highest shoot length of 54.3 ± 3.53 mm. Furthermore, 4.4 µM 6-BA combined with 0.5 µM IBA was superior for the highest shoot number with 3.4 ± 0.49 after 4-wk culture. Cytogenetic analyses indicated that all propagated plants have the same DNA ploidy level (x) and chromosome number ($2n = 18$) compared with the mother plants. After 4 wk, the rooting percentage achieved 100% in all tested rooting media. MS medium supplemented with 2.7 µM NAA favored the highest root number, root length, and secondary root number with 3.44 ± 0.5, 170.1 ± 4.91 mm, and 26.1 ± 1.65, respectively. Rooted plantlets were initially acclimatized and then transferred in greenhouse conditions. This method could be evaluated for *ex situ* conservation of rare endemic and endangered plant species.

Keywords *Tripleurospermum fissurale* · Endangered species · *Ex situ* · Cytogenetic stability · Micropropagation

Introduction

In recent years, in order to create an effective plant diversity conservation program, it is necessary to develop plant-specific protection methods such as *in situ* and *ex situ* by conservation programmers (Kurt and Erdag 2009). *In vitro* culture methods serve *ex situ* protection from these conservation programs with effective solution approaches in the short, medium, and long term (Fay 1994; Kurt and Erdag 2009; Krogstrup *et al*. 1992). The evaluation of *in vitro* propagation techniques has gained great importance and momentum in recent years, especially for the rapid cultivation of species that grow in a narrow habitat with limited reproductive capacity, and therefore are in danger of extinction (Fay 1992; Kurt and Erdag 2009).

Mature seeds are generally preferred as productive starting explant to establish cultures within *in vitro* culture studies because they are compatible with the genetic structure of the narrow target population to be protected (Alves *et al*. 2006). The seed germination within *in vitro* culture also contributes to obtaining a large number of aseptic explants to be preferred in subsequent micropropagation studies thanks to subculture treatments. (Mercier and Kerbauy 1997; Kurt and Erdag 2009).

Tripleurospermum Sch.Bip. is a small genus of the tribe Anthemideae belonging to the Asteraceae family. It is mainly spread in Europe, temperate Asia, North America, and North Africa and is represented by nearly 40 species (Oberprieler *et al*. 2007). The genus includes 32 taxa in the Turkish flora (Ozbek and Onayli 2020; Inceer and Ozcan 2021). The endemism rate of *Tripleurospermum* is 50% in Turkey (Inceer and Ozcan 2021). The endemic taxa of *Tripleurospermum* have also been used pharmaceutically such as anti-inflammatory, antiseptic, antifungal, antibacterial, antiulcer, and antioxidant activity (Ghassemi-Dehkordi *et al*. 2003; Amin *et al*. 2004; Minaiyan *et al*. 2006; Hosseini *et al*. 2007; Chehregani *et al*. 2010; Erdogan *et al*.

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2013; Zeljkovic *et al*. 2015; Colak *et al*. 2017). In addition to all these, species belonging to this genus are used in traditional folk medicine such as relieving headache and toothache, regulating blood sugar, wound healing, cough, asthma, respiratory tract problem, and removing acne (Cansaran *et al*. 2007; Altundag and Ozturk 2011; Tetik *et al*. 2013; Han and Bulut 2015; Mohammadi *et al*. 2016; Nadiroglu *et al*. 2019). However, some endemic *Tripleurospermum* species are endangered due to several anthropogenic pressures such as chemical pollution, habitat fragmentation, grazing, and all kinds of activities within the scope of nature tourism (Ekim *et al*. 2000).

Tripleurospermum fissurale (Sosn.) E.Hossain is a rare and endemic species for Turkey since it is only distributed in Artvin and Erzurum Provinces (Enayet Hossain 1975; Inceer 2012). It grows on rocky crevices and roadsides at 300 to 600 m a.s.l. (above sea level). *Tripleurospermum fissurale* has pharmaceutical value (Colak *et al*. 2017). However, this species is directly the other indirectly threatened by several anthropogenic pressures such as habitat fragmentation, soil erosion, and road and dam constructions. These anthropogenic pressures cause a decrease in population size and density of this species. According to Ekim *et al*. (2014), *T. fissurale* is placed in the endangered (EN) category.

Within the genus *Tripleurospermum*, only one endemic species of *Tripleurospermum* has been multiplied and conserved using *in vitro* culture technique: *Tripleurospermum insularum* Inceer & Hayırlıoglu-Ayaz (Inceer *et al*. 2022). Since there is no detailed report aimed at the conservation of *T. fissurale*, the present study was planned to develop suitable methods of achene germination as well as efficient and required conditions for biotechnological seedling development and to confirm cytogenetic fidelity of micropropagated plants with flow cytometric analysis (FCM) and chromosome counting.

Material and methods

Plant material and achene germination The mature achenes were obtained from collection of *T. fissurale* (Turkey: A8 Artvin, Yusufeli, Inceer 724, at herbarium of Karadeniz Technical University Biology, KTUB). Two basal media Murashige and Skoog (MS) (Murashige and Skoog 1962) (Duchefa Biochemie, Haarlem, The Netherlands) and Gamborg's B-5 (B5) (Gamborg *et al*. 1968) (Duchefa Biochemie, Haarlem, The Netherlands), each supplemented with 4.7 µM kinetin (Sigma-Aldrich, St Louis, MO), were tested to determine the optimum basal culture media for achene germination. The percentage of germination was calculated for each treatment after 30 d of incubation.

Experimental-Sterilization Before surface sterilization, the mature achenes were deeply washed with tap water for 30 min on a magnetic stirrer, followed by quickly subjecting them to a second pretreatment with 70% (*v/v*) ethanol (EtOH) for 30 s. After these processes, ethanol was completely removed from the environment, and these achenes were carefully and deeply disinfected with 20% commercial bleach (Domestos) for 10 min. Finally, removal of bleach residues was carried out in 3 repetitions for 15 min in each application with sterile deionized water in a sterile cabinet. Magenta tissue culture vessel with a size of 98.5 × 59 mm (Sigma-Aldrich, St Louis, MO) containing approximately 30 mL of basal medium (including vitamins) was preferred for culturing. Cultured achenes were kept in growth chamber until the beginning of germination.

Shoot proliferation Nodal segments after the third subculture were excised from the healthy shoots of seedlings and vertically placed on MS basal medium. This basal medium was supplemented with 2% (*w/v*) sucrose (Duchefa Biochemie, Haarlem, the Netherlands) and 0.8% (*w/v*) phyto agar (Duchefa Biochemie, Haarlem, the Netherlands). Then different concentrations of plant growth regulators (PGRs) namely 4.4 µM 6-benzylaminopurine (6-BA), 4.7 µM kinetin (KIN), 4.9 µM 6-(y,y-dimethylallylamino)-purine (2iP), 4.5 µM thidiazuron (TDZ), and 4.6 µM zeatin as cytokinin and in combination with indole-3-butyric acid (IBA), indole-3-acetic acid (IAA), or α-naphtaleneacetic acid (NAA), at 0.5 µM each, as auxin were individually added to these media to support shoot multiplication. Among the PGRs supplied from Sigma (Sigma-Aldrich, St Louis, MO) that were used in this study, only 6-BA and NAA were added in media before autoclaving. The other ones were sterilized with 0.22-µm filters and added to the cooled (nearly 40ºC) media after autoclaving. The pHs of the media were readjusted to 5.8 before autoclaving with 1 M HCl or 1 M NaOH. Climate room conditions were adjusted to be 24 ± 2 °C and a 16/8 h photoperiod at a photosynthetic flux of 50 µmol m^{-2} s^{-1}. The lighting process was carried out with cool daylight fluorescent lamps (LUMILUX, HE 21 W/840, OSRAM, Italy). The subculturing period for this species was ideally every 4 wk. After culturing for 1 mo, the proliferation was carefully assessed by evaluating the number of shoots *per* explant, length of shoots, node number on each of the shoots, callus formation, and plant quality (internode length and shoot thickness).

Cytogenetic analyses For DNA ploidy level (*x*) determination with FCM, the young leaves obtained from *in vitro per* treatment and *in vivo* plants were chopped using a razor blade in 1 mL of woody plant buffer (Loureiro *et al*. 2007). The suspension was filtered through a 30-µm nylon mesh, and 50 µg /mL^{-1} DNase-free RNase was added to the nuclei

suspension and then stained with 50 µg mL^{-1} propidium iodide. Samples were analyzed using a BD Accuri C6 flow cytometer. At least 10,000 nuclei were measured from each sample. Five individuals were measured two times on two different days *per* treatment (Inceer *et al*. 2022). The DNA ploidy level was then estimated according to Doležel *et al*. (2007).

Chromosome counts were carried out in rooted shoots. For this purpose, firstly, well-developed root tips were pretreated with 2 mM 8-hydroxyquinoline for 6 h at room temperature. Later, the root tips were fixed in ethanol-glacial acetic acid (3:1) for at least 24 h at 4 °C, hydrolyzed in 1 M HCl at 60 °C for 12 to 13 min, and stained with Schiff's reagent for 2 to 3 h at room temperature, respectively (Inceer and Beyazoglu 2004). The squashes were made in 45% acetic acid and the preparations were mounted in Entellan. At least five well-spread metaphase plates from five individuals were used for chromosome counting, and were then photographed by means of a Leica DM 1000 microscope (Leica Microsystems, Wetzlar, Germany).

Root induction The proliferated shoots and elongated shoots (≥ 20 mm) were rooted on MS medium supplemented with 2.5 µM IBA, 2.9 µM IAA, and 2.7 µM NAA and were used as an auxin source for root induction. Cytokinin/auxin-free MS medium was evaluated as a control. Different rooting media were used for root induction of each shoot obtained from different shooting media. Rooting percentage was also evaluated *via* rooting percentage of microshoots, number of roots, length of roots, number of secondary roots (the lateral roots on the longest root), and callus formation *per* explant after 4 wk. Each experiment was performed in triplicate.

Acclimatization Rooted plantlets were rinsed to remove medium residue with tap water and the roots were cut to leave a root length of 30 mm before being transferred to *ex vitro* conditions. Finally, rooted microshoots were transferred to 71 × 71 mm plastic pots containing the vermiculite supplemented with Hoogland solution (pH 5.8) and acclimatized by gradually lowering the humidity over 4 wk in climate room. Climate room conditions were adjusted to be 24 ± 2 °C and a 16/8 h photoperiod at a photosynthetic flux of 50 µmol m^{-2} s^{-1}. The lighting process was carried out with cool daylight fluorescent lamps as the abovementioned shoot proliferation studies. At the end of the 4th wk, viability percentage of microshoots was measured, and surviving plantlets were transferred to greenhouse conditions. The humidity and temperature were adjusted to 80 to 85% and 26 ± 2 °C in the greenhouse, respectively. Only peat soil was chosen in the greenhouse conditions. Peat and forest soil (1:1 (*v/v*)) were preferred for transferring the plantlets to the botanical garden.

Statistical analysis Five achenes were placed *per* Magenta and four Magentas were used *per* treatment. For all shoot proliferation studies, four nodal explants were cultured in one glass Magenta culture vessel, and six vessels were preferred for each treatment. For each rooting treatment, sixteen healthy shoots were tested so as to be four shoots in one Magenta. Each treatment was repeated as triplicates in achene germination, shoot proliferation, and root induction studies. The statistical analyses were carried out to evaluate the shoot proliferation and root induction using SPSS version 21. Duncan's multiple range tests from one-way analysis of variance were employed to determine the statistical significance of differences among the means in shoot proliferation and root induction.

Results

Achene germination Preliminary experiments have shown that the cytokinin-free MS medium was insufficient in terms of the germination success and shoot length of the achenes (data not shown). For achene germination, therefore, the basal medium was supplemented with a cytokinin. The first achenes germination appeared at the end of the 5th and 6th days after inoculation. Maximum germination percentage was achieved after the 16th and 18th days in MS and B5 media, respectively. No bacterial or fungal contamination was observed during the germination period. MS containing 4.7 µM KIN was found to be superior to B5 basal medium since the former supplied 60 ± 5.0% achene germination percentages. The achene germination number was found to be statistically significant between both media ($P < 0.05$). Based on these results, MS medium was chosen in repeated subsequent shoot proliferation of rare endemic endangered *T. fissurale*.

Shoot proliferation The maximum shoot number was recorded on MS medium containing 4.4 µM 6-BA and 0.5 µM IBA with 3.4 ± 0.49 *per* explant. On the other hand, IAA applications in combination with different cytokinins were less effective than IBA and NAA applications in terms of shoot number. Statistical analysis demonstrated significant differences between 6-BA, control, and other tested cytokinins in terms of the shoot number ($P < 0.05$).

Irrespective to shoot number, 2iP combined with IBA showed higher performance with zeatin than those of other tested media in terms of the shoot elongation (Table 1). The 2iP and IBA combination gave the highest shoot length with 54.3 ± 3.53 mm *per* explant (Fig. 1*d*). Although the aforesaid combination was very effective in terms of shoot lengths, leaf number, leaf size, and leaf width varied significantly depending on the type of auxin (Fig. 1*a–p*). If all of these important differences were left aside, it could be said that

Table 1. Effects of different cytokinin in the presence of IBA, IAA and NAA (0.5 µM) combination and control group on the shoot proliferation as well as DNA ploidy level of *Tripleurospermum fissurale* (Sosn.) E.Hossain

Cytokinin (µM)						Auxin (µM)								
	KIN	6-BA	2iP	TDZ	ZEA	IBA	IAA	NAA	Shoot number per explant	Shoot length per explant (mm)	Node number per explant	Callus rate (%)	DNA ploidy level (*x*)	DNA-peaks ratio	CV (%)
	4.7					0.5			2.8 ± 0.48b	31.9 ± 2.83e	6.1 ± 0.83d	51.4 ± 2.4e	2*x*	1.03	2.98
		4.4				0.5			3.4 ± 0.49a	31.6 ± 2.45e	7.9 ± 0.72b	45.8 ± 2.4f	2*x*	0.99	3.47
			4.9			0.5			2.7 ± 0.48b	54.3 ± 3.53a	7.3 ± 0.96c	26.4 ± 2.4 h	2*x*	0.98	2.84
				4.5		0.5			1.6 ± 0.50de	25.9 ± 1.81 h	3.3 ± 0.44i	81.9 ± 4.8b	2*x*	1.08	3.51
					4.6	0.5			2.7 ± 0.48b	47.9 ± 2.89b	5.9 ± 0.68de	13.9 ± 2.4j	2*x*	1.03	3.05
	4.7						0.5		1.5 ± 0.51de	29.8 ± 2.77 g	3.7 ± 0.64i	44.4 ± 2.4 fg	2*x*	1.06	2.98
		4.4					0.5		1.6 ± 0.49de	24.7 ± 2.37hi	4.5 ± 0.51 g	66.7 ± 4.2d	2*x*	1.20	2.41
			4.9				0.5		1.5 ± 0.51de	24.9 ± 1.50hi	4.3 ± 0.53 g	30.6 ± 2.4 h	2*x*	1.11	2.75
				4.5			0.5		1.3 ± 0.48e	23.4 ± 1.96i	3.3 ± 0.44 h	75.0 ± 4.2c	2*x*	1.12	3.65
					4.6		0.5		1.8 ± 0.44d	31.4 ± 2.52ef	5.2 ± 0.56f	30.6 ± 2.4 h	2*x*	1.12	2.47
	4.7							0.5	2.3 ± 0.55c	37.6 ± 2.14d	8.0 ± 0.69b	40.3 ± 4.8 g	2*x*	0.94	2.89
		4.4						0.5	2.7 ± 0.48b	30.0 ± 2.43 fg	9.2 ± 0.96a	54.2 ± 4.2e	2*x*	0.98	3.26
			4.9					0.5	2.2 ± 0.59c	39.1 ± 2.68c	8.0 ± 0.72b	26.4 ± 2.4 h	2*x*	0.97	2.64
				4.5				0.5	1.5 ± 0.51de	16.5 ± 1.43j	2.6 ± 0.58j	100.0 ± 0.0a	2*x*	0.87	2.90
					4.6			0.5	2.3 ± 0.55c	40.7 ± 3.86c	5.6 ± 0.65e	75.0 ± 4.2c	2*x*	1.04	3.15
Control (*in vitro* plants without PGRs)									1.7 ± 0.48d	40.6 ± 3.50c	8.3 ± 0.87b	nd	2*x*	1.08	3.34

Data were recorded 4 wk after the culture and represent a total of three replicates of 24 plants *per* treatment on MS for shoot proliferation and 10 plants *per* treatment for nuclear DNA analysis. Values having the same *letter(s)* in the same *column* are not significantly different according to Duncan's multiple range test at $P < 0.05$ for shoot proliferation. *CV* coefficient of variation, *nd* not detected, *KIN* kinetin, *6-BA* 6-benzylaminopurine, *2iP* 6-(y,y-dimethylallylamino)-purine, *TDZ* thidiazuron, *ZEA* zeatin, *IBA* indole-3-butyric acid, *IAA* indole-3-acetic acid, *NAA* α-naphthaleneacetic acid.

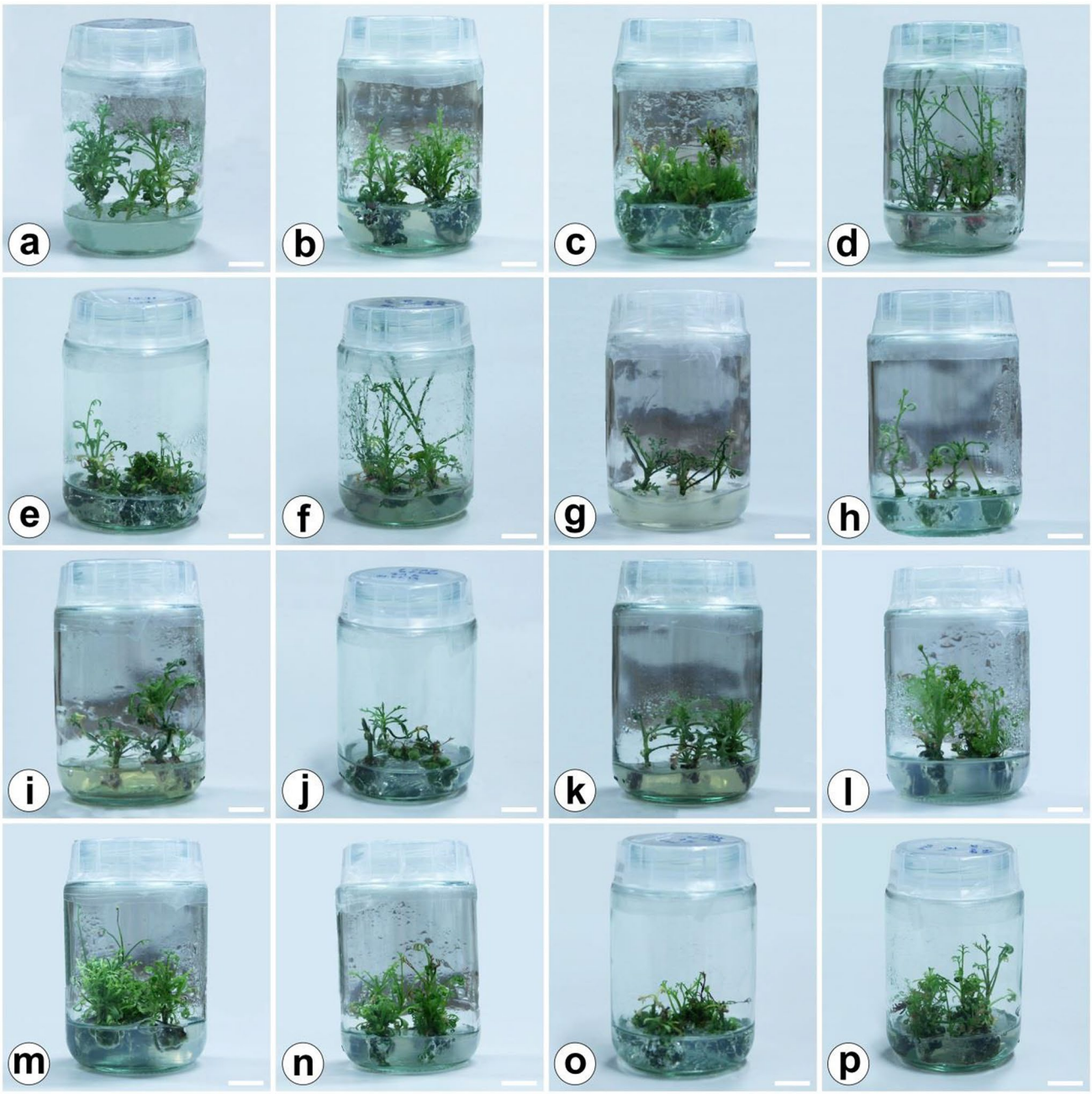

Figure 1. Shoot multiplication of *Tripleurospermum fissurale* (Sosn.) E.Hossain. (*a*) Control, (*b*) 4.7 + 0.5 µM KIN + IBA, (*c*) 4.4 + 0.5 µM 6-BA + IBA, (*d*) 4.9 + 0.5 µM 2iP + IBA, (*e*) 4.5 + 0.5 µM TDZ + IBA, (*f*) 4.6 + 0.5 µM ZEA + IBA, (*g*) 4.7 + 0.5 µM KIN + IAA, (*h*) 4.4 + 0.5 µM 6-BA + IAA, (*i*) 4.9 + 0.5 µM 2iP + IAA, (*j*) 4.5 + 0.5 µM TDZ + IAA, (*k*) 4.6 + 0.5 µM ZEA + IAA, (*l*) 4.7 + 0.5 µM KIN + NAA, (*m*) 4.4 + 0.5 µM 6-BA + NAA, (*n*) 4.9 + 0.5 µM 2iP + NAA, (*o*) 4.5 + 0.5 µM TDZ + NAA, (*p*) 4.6 + 0.5 µM ZEA + NAA. *Bars* = 2 cm. KIN, kinetin; 6-BA, 6-benzylaminopurine; 2iP, 6-(y,y-dimethylallylamino)-purine; TDZ, thidiazuron; ZEA, zeatin; IBA, indole-3-butyric acid; IAA, indole-3-acetic acid; NAA, α-naphthaleneacetic acid.

2iP in combination with IBA was the best treatment for obtaining highest shoot length ($P < 0.05$).

As it happened in the shoot number, the lowest shoot length was obtained from IAA applications which were combined with five different cytokinins. Node number was the most important parameter for shoot proliferation, and the highest node number was obtained from MS medium supplemented with 4.4 µM 6-BA and 0.5 µM NAA (9.2 ± 0.96 *per* explant) whereas the lowest was from IAA applications. When compared, a significant difference could be seen between 6-BA and all other applications in terms of node number ($P < 0.05$).

Only the control group did not cause callus formation. All TDZ applications combined with three different auxin types gave the highest callus formation percentage. TDZ with NAA, IAA, or IBA combinations gave rise to 100%, 81.9%, and 75% callus formation, which was an undesirable phenomenon in terms of the rooting studies.

DNA ploidy level and chromosome number The results of FCM analysis are presented in Table 1. Coefficients of variation range from 2.41 and 3.65% (with mean value 3.02%) indicating reliable and reproducible estimates. According to the result obtained, there was no variation in DNA ploidy level of the propagated plants. The results obtained from chromosome counting were given in Table 2 and Fig. 3. As seen in Table 2 and Fig. 3, the somatic chromosome number of all propagated plants was $2n = 2x = 18$.

Root induction Well-developed and healthy shoots (> 20 mm) were excised from the shoot culture media and transferred to MS media. After 4 wk, MS basal media supplemented with IBA or NAA stimulated the rooting more efficiently than the control and IAA treatments. The first root formation was observed in NAA applications after 18 d as well as the longest root length. At the end of the 30 d, 100% rooting success was achieved in all tested media. In terms of the evaluated parameters in rooting studies, NAA was found to be more effective than all the other tested auxins and control (Fig. 2*a*). The highest root number, root length, and secondary root number were calculated as 3.44 ± 0.50, 170.1 ± 4.91 mm, and 26.1 ± 1.65, respectively (Table 2). IAA and control applications were less successful in rooting of *T. fissurale* compared to other applications.

Acclimatization and *ex situ* collection To determine the viability percentage of microshoots in *ex vitro* conditions, the well-developed *in vitro* rooting plantlets were transferred in plastic pots containing the vermiculite supplemented with Hoogland solution (pH 5.8). The top of the pots were covered with plastic bags to provide humidity. To keep the vitality of *in vitro* rooting plantlets high in *ex vitro*, the pots were transferred in climate room conditions (24 ± 2 °C, 16/8 photoperiod, and 90% humidity). Rooting plantlets showed the highest viability percentage (83.3%) with 55.7% shoot and 120.2% root elongation in climate room conditions. In both the greenhouse and the botanical garden, the highest survival percentage was achieved by plantlets obtained from

Table 2. The effects of different auxin types on *in vitro* rooting and chromosome number (2*n*) in *Tripleurospermum fissurale* (Sosn.) E.Hossain

PGRs (μM)	Root number (no/plant)	Root length (mm)	Secondary root number (no/plant)	2*n*
CONTROL	2.51 ± 0.42c	152.7 ± 7.43b	17.9 ± 0.95c	18
2.5 IBA	3.07 ± 0.35b	154.0 ± 5.20b	19.6 ± 1.67b	18
2.9 IAA	2.86 ± 0.33b	167.0 ± 6.36a	17.5 ± 1.46c	18
2.7 NAA	3.44 ± 0.50a	170.1 ± 4.91a	26.1 ± 1.65a	18

Data were recorded on the 4 wk after the culture and represent a total of three replicates of 16 plants *per* treatment for root induction

Values having the same *letter(s)* in the same *column* are not significantly different according to Duncan's multiple range test at $P < 0.05$

IBA indole-3-butyric acid, *IAA* indole-3-acetic acid, *NAA* α-naphthaleneacetic acid.

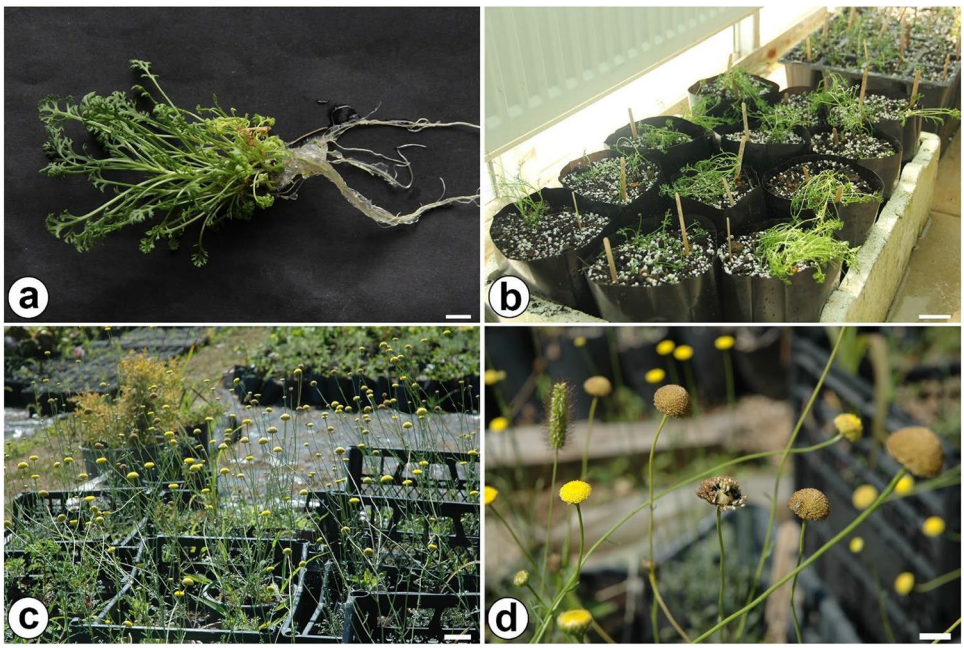

Figure 2. Micropropagation of *Tripleurospermum fissurale* (Sosn.) E.Hossain. (*a*) 2.5 μM IBA (indole-3-butyric acid) on *in vitro* rooting of *T. fissurale* from shoot-bud culture-derived seedlings, (*b*) greenhouse conditions, (*c*, *d*) botanical garden conditions. *Bars* = a: 1 cm, b–c: 5 cm, d: 2 cm.

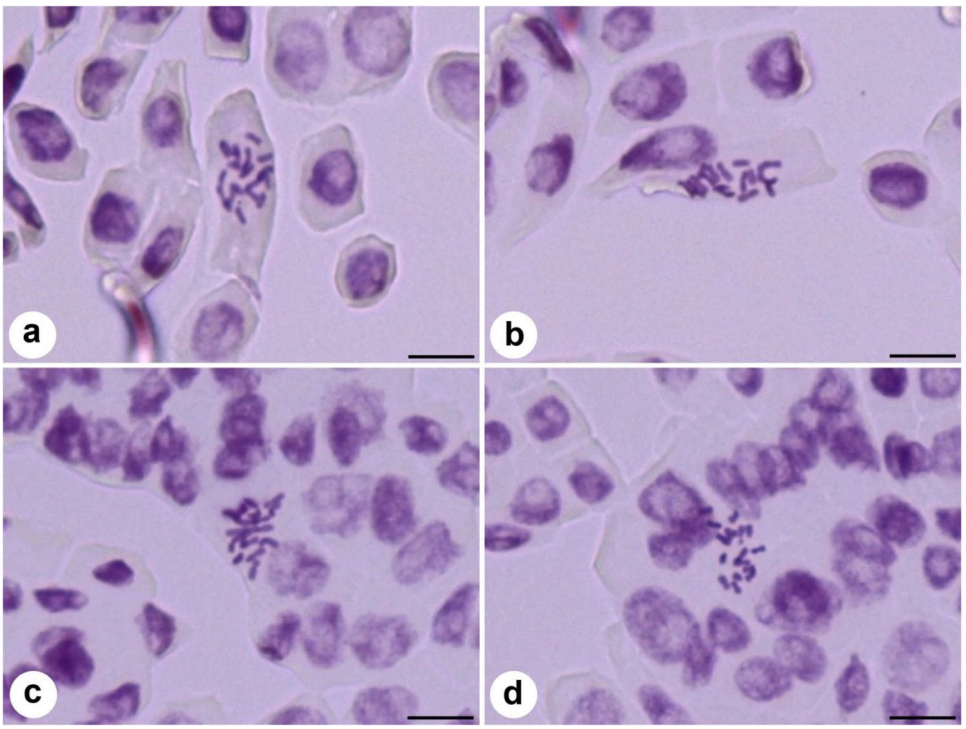

Figure 3. Mitotic metaphase chromosomes of propagated *Tripleurospermum fissurale* (Sosn.) E.Hossain. plants. (*a*) Chromosomes of control plant on Murashige and Skoog (MS) medium without PGRs ($2n = 18$), (*b*) chromosomes of *in vitro* plant on MS medium supplemented with 2.5 µM IBA ($2n = 18$), (*c*) chromosomes of *in vitro* plant on MS medium supplemented with 2.9 µM IAA ($2n = 18$), (*d*) chromosomes of *in vitro* plant on MS medium supplemented with 2.7 µM NAA ($2n = 18$). *Scale bars* = 10 µm. IBA, indole-3-butyric acid; IAA, indole-3-acetic acid; NAA, α-naphthaleneacetic acid.

MS medium supplemented with IBA. At the end of 4 wk, healthy and well-developed acclimatized plantlets were relocated again to greenhouse conditions. The percentage of survival was calculated as approximately 90% (Fig. 2*b*). On the contrary, the survival rate of the plantlets in the botanical garden was determined as about 81%.

Plants were easily acclimatized to the open air-field plot in the botanical garden, as well. After 30 d, they developed new leaves and grew considerably (Fig. 2*c*). All the plants formed buds, bloomed, and produced achenes within the first season (Fig. 2*d*). The *ex situ* collection of *T. fissurale* has been established at the end of spring 2019 and completed during the summer of the same year (Fig. 3).

Discussion

In vitro methods have recently been used to propagate the economic, medicinal and aromatic, and endemic and endangered plant species, aiming to provide several advantages, such as using a more convenient and controlled culture conditions, faster and easy production, obtained disease-free plants, supplying the more effective clonal propagation, and shortening the growth cycle time (Ostrolucká *et al*. 2004). In connection with this, *ex situ* conservation studies have gained increased momentum, especially endemic and endangered species in the world; and in this context, the methods of plant biotechnology have often been used for increasing the number of these plant species protecting the ancestral character. Many researchers have studied the members of different plant families to identify *in vitro* growth protocols and to perform *ex situ* protection (Ambrožič Dolinšek *et al*. 2016; Nalawade *et al*. 2016; Upadhye *et al*. 2016; Cüce 2021). Although a few researchers have studied on the members of the family Asteraceae in tissue cultures (Pace *et al*. 2009; Kher *et al*. 2014), *ex situ* conservation studies on the genus *Tripleurospermum* are limited to only *T. insularum* (Inceer *et al*. 2022). Therefore, *ex situ* conservation of the *T. fissurale via in vitro* production techniques was remarkable and noteworthy.

Different sterilization methods, employed basal media, utilization of plant growth regulators (PGRs), and different culture conditions can be found elsewhere for micropropagation of different plant species (Mallón *et al*. 2010; De resende *et al*. 2016; Cüce *et al*. 2017; Bekircan *et al*. 2018). Bouhouche and Ksiksi (2007) carried out NaOCl surface sterilization methods on *Teucrium stocksianum* Boiss., an endangered and valuable medicinal plant, and obtained successful germination rate within 15 d using MS medium, which is the same with the present study's germination results.

The results obtained from *in vitro* achene germination indicated that MS medium was more efficient than B5 medium. This finding was an agreement with the previous report for *T. insularum* (Inceer *et al*. 2022).

The present results showed that *T. fissurale* had a relatively low germination rate (60%), which was in line with

the previous *in vivo* germination test for chromosome counting of this species (Inceer and Hayirlioglu-Ayaz 2014). On the other hand, its seedlings were healthy and suitable for obtaining plants.

The MS medium was always the preferable basal medium containing KIN, 6-BA, 2iP, and TDZ as the cytokinins and IBA, IAA, and NAA as auxins in the *in vitro* studies regarding the other members of the Asteraceae family (Kurt and Erdag 2009; Corral *et al*. 2011; Kher *et al*. 2014; Shinde *et al*. 2017). In addition to seed germination, MS containing 6-BA was the most effective medium in terms of the shoot proliferation of different plant species (De Souza *et al*. 2007; Ambrožič Dolinšek *et al*. 2016; Valletta *et al*. 2016). Although MS medium with 6-BA was more effective in the production of shoot number, the combination of 2iP and IBA gave the highest shoot length in the present study. TDZ and any auxin combinations were not preferable as they gave the lowest shoot number. The present study's findings were in agreement with the previous results concerning the highest multiple shoot formation (Mallón *et al*. 2010; Ambrožič Dolinšek *et al*. 2016; Shinde *et al*. 2017).

The single study on the genus *Tripleurospermum* was conducted by Inceer *et al*. (2022). These researchers preferred the same concentrations and combinations of cytokinins and auxins applied in this study. These researchers found that MS medium supplemented with ZEA and IAA was more effective in terms of the highest shoot number. The same researchers again obtained the highest shoot length in MS medium containing the combination of zeatin and IAA or control group. All these results revealed differences between species belonging to the same genus.

Callus formations were detected between 13.9 and 100% in all tested PGRs on shoot multiplication. These data revealed that TDZ applications combined with IBA, IAA, or NAA were higher in terms of callus formation ability in the *T. insularum*. The clearest indicator of this result was that the highest callus formation was 100% and obtained from cultures grown on medium containing TDZ and NAA. As mentioned above, callus formation percentages vary depending on the explant type, PGR combination, and concentration used in *in vitro* studies on other members of Asteraceae (Sánchez-Ramos *et al*. 2018; Abu-Darwish *et al*. 2022). Besides, the fact that the highest rate of callus formation was obtained from MS medium containing TDZ with 100% in the study on *T. insularum* was the most important evidence of the data this present study obtained (Inceer *et al*. 2022).

Generally, researchers employed different concentrations of IBA, IAA, and NAA for *in vitro* rooting studies of the members of Asteraceae (Pace *et al*. 2009; Emek and Erdag 2013; Cüce *et al*. 2019; Inceer *et al*. 2022). Dhaka and Kothari (2005) used IBA (2.5, 5.0, and 10.0 µM), IAA (2.9, 5.8, and 11.6 µM), and NAA (2.7, 5.4, and 10.8 µM) for *in vitro* rooting studies of *Eclipta prostrata* L. MS containing 5.0 µM IBA was given the highest results with 100% rooting success according to the these researchers' study. The data obtained from this present study showed differences to the Dhaka and Kothari' results. In another study carried out on *Achillea millefolium* L. (Asteraceae), IBA, IAA, and NAA were used at different concentrations (Shatnawi 2013). These researchers reported that the low NAA concentrations gave the highest rooting percentage and root length while root number was supplied by MS with 5.9 µM IBA. This present study's findings also supported these results.

As far as this study's results relating to rooting success were concerned, there was a prerequisite to optimize auxin treatment for *T. fissurale* seedlings. MS with 2.5 µM IBA or 2.7 µM NAA could be evaluated as the most promising media for this purpose. All data presented here were comparable with those of Evenor and Reuveni (2004), De Souza *et al*. (2007), and Arya and Patni (2013). In the case of Evenor and Reuveni (2004), MS without PGRs was the most favorable medium for the highest root length of *Achillea filipendulina* Lam. That was completely different from this present study's findings. Besides, according to their findings, rooting percentage was 64%, which was far below the present study's findings having 100% rooting success. Moreover, according to Corral *et al*. (2011) and Arya and Patni (2013), MS with IBA was most suitable in terms of the highest root number. These results differed from the present study's findings which show NAA was the best for this purpose. Last, but not least, it should be pointed out that results were subject to changes according to species studied, the cultivar of the same species, the test medium, and the auxins, all being employed in *in vitro* rooting studies on Asteraceae (Arya and Patni 2013).

It was known that *T. fissurale* is a diploid species with $2n = 18$ chromosomes (Inceer and Hayirlioglu-Ayaz 2010). The results of cytogenetic analyses indicated that the propagated plants were diploid ($2n = 18$) as in mother plants. This result showed that there is no change in genetic stability of the propagated plants.

Conclusion

In this work, an efficient protocol was developed for micropropagation of the rare and endemic *T. fissurale* endangered species in the world. Achene germination, shoot proliferation, root induction, and acclimatization protocol were completed with high success percentages. The protocol presented here describes micropropagation of *T. fissurale* with cytogenetic fidelity. Hence, it may be used for conservation activities of this species.

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