In vitro propagation and cytogenetic stability of Tripleurospermum insularum (Asteraceae) – a critically endangered insular endemic species from Turkey

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\textbf{Abstract}

Tripleurospermum insularum (Asteraceae) is a critically endangered (CR) insular endemic species in Turkey and is facing high risk of extinction. Here, a rapid and efficient \textit{in vitro} propagation protocol using nodal segments obtained from seedling shoots cultured on Murashige and Skoog (MS) basal medium supplemented with different plant growth regulators (PRGs) was developed to conserve \textit{T. insularum}. Besides, the cytogenetic fidelity of propagated plants was tested with DNA ploidy level using flow cytometry (FCM) as well as chromosome counting. The highest shoot number and length of shoot per explant were achieved in MS medium containing 4.6 \( \mu \text{M} \) zeatin (ZEA) and 0.5 \( \mu \text{M} \) indole-3-acetic acid (IAA). No variation in DNA ploidy level (2n) and somatic chromosome number (2n = 18) of all propagated plants were observed. \textit{In vitro} rooting of shoots was achieved at 100\% efficiency in the medium supplemented with 2.9 \( \mu \text{M} \) IAA. The rooted plantlets were transferred \textit{ex vitro} with 74\% survival. This is the first report of a successfully developed micropropagation protocol for \textit{ex situ} conservation of \textit{T. insularum}.

\textbf{Introduction}

The International Union for Conservation of Nature (IUCN) Red Lists indicate that insular species have high extinction risk due to their low competitive ability and restricted distribution as well as anthropogenic pressure and/or environmental factors (Gray 2018). In particular, the insular species in the Mediterranean Basin are highly vulnerable (Cañadas et al. 2014; Médail 2017; Cogoni et al. 2019). Tripleurospermum Sch.Bip. is a typically Mediterranean genus of the tribe Anthemideae of the family Asteraceae with ca. 40 species that is mainly distributed in Europe, temperate Asia, North America and North Africa (Oberprieler et al. 2007). Our recent molecular phylogenetic analyses based on internal transcribed spacer (ITS) and external transcribed spacer (ETS) of nuclear ribosomal DNA (nrDNA) regions indicate that \textit{Tripleurospermum} is monophyletic (Inceer et al. 2018). Within the genus, two large highly supported clades including diploids and polyploids are distinguished, and ploidy levels are also significantly different between the clades (Inceer et al. 2018).

\textit{Tripleurospermum} is particularly abundant and diverse in Turkey, its main center of diversity, with ca. 32 taxa and a rate of endemicism of 50\% in the country (Inceer and Ozcan 2021). Some species, such as \textit{T. heterolepis} (Freyr & Sint.) Bornm., \textit{T. insularum} Inceer & Hayırlıoglu-Ayaz, and \textit{T. ziganaense} Inceer & Hayırlıoglu-Ayaz are also stenoendemic (i.e. species with a restricted distribution area).

\textit{Tripleurospermum insularum} is an insular endemic species with a highly restricted geographic range in Turkey; it occurs as a single population in Gökçeada, one of the Aegean Islands (Inceer and Hayırlıoglu-Ayaz 2014). \textit{T. insularum} grows in open places and on rocky slopes at an altitude of 30 m a.s.l., and the dominant type of vegetation in the area is phrygana (Inceer and Hayırlıoglu-Ayaz 2014). According to Inceer and Hayırlıoglu-Ayaz (2014), this narrow endemic species is isolated both geographically and ecologically from other Turkish \textit{Tripleurospermum} taxa except for \textit{T. roselium} (Boiss. & Orph.) Hayek var. \textit{album} E.Hossain, indicating its origin through allopatric speciation.

It is not clear the relationships between \textit{T. insularum} and other taxa. This species resembles \textit{T. auriculatum} (Boiss.) Rech.f., which is not found in the Turkish flora. Besides, molecular phylogenetic analyses indicate a close affinity of diploid \textit{T. insularum} (2n = 18) to diploid \textit{T. parviflorum} (Willd.) Pobed. (2n = 18) and diploid \textit{T. conoclinium} (Boiss. & Bal.) Hayek (2n = 18) (Inceer et al. 2018, and references therein). In particular, \textit{T. parviflorum} is the most widespread among these species. According to the terminology of Siljak-Yakovlev and Peruzzi (2012), \textit{T. insularum} can be considered schizoendemic.

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**T. insularum** is an annual herb, reaching a high of 8–14 cm. Stems are two to many, unbranched, occasionally with two small branches near the base. Leaves are one to three-pinnatisect, laciniae are thin and linear; bottom leaves are petiolated; cauline leaves are sessile. Capitula are heteromalous, radiate. Receptacle is ovate-conical. Ray flowers are female, limbs are white, broadly eliptic and shallowly three-lobed at apex. Disc flowers are numerous, hermaphroditic and corolla lobes eglandular at tips. Achenes are shortly oblong, ± incurved, black or dark brown at maturity, copiously mucilaginous; dorsal side is smooth, ventral side is three-ribbed and ribs are thickened. Corona is absent (Inceer and Hayırlıoğlu-Ayaz 2014).

Like other endemic taxa of *Tripleurospermum* (Altundag and Ozturk 2011; Han and Bulut 2015; Mohammadi et al. 2016), *T. insularum* has a pharmaceutical potential with rich phenolics compounds and potent antioxidant properties (Zeljkovic et al. 2015; Colak et al. 2017).

According to Inceer and Hayırlıoğlu-Ayaz (2014), *T. insularum* is classified within the critically endangered (CR) category of the IUCN. The IUCN Red List extinction data of this species are associated with low competitive ability and anthropogenic factors, such as overgrazing, trampling and fragmentation. The population is, therefore, facing extinction in the near future, unless in situ and/or ex situ conservation strategies are adopted as soon as possible.

**In vitro** propagation is commonly used for conservation of threatened endemic and/or rare species. Particularly, it is important for the species having small populations (Gonçalves et al. 2010; Mallón et al. 2011). Besides, *in vitro* culture is employed as a means of producing materials from medicinal plants (Wu et al. 2021). On the other hand, the genetic fidelity of propagated plants is required to test the success of *in vitro* propagation protocol for conservation programs because of somaclonal variations associated with stress conditions during tissue culture (Bairu et al. 2011; Adhikari et al. 2014; Slazak et al. 2015; Corduk et al. 2018; Ulvrova et al. 2021). The somaclonal variations of propagated plants can be detected with cytogenetic analyses based on chromosome counting and/or flow cytometry (FCM) as well as molecular markers, such as amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), simple sequence repeats (SSRs), inter-SSRs (ISSRs) and single-nucleotide polymorphisms (Orbovic et al. 2008; Corduk et al. 2018; Park et al. 2020; Ulvrova et al. 2021).

FCM offers a feasible technique for fast and large-scale analysis of DNA content and cytogenetic stability of plants (Doležel et al. 2007a; Sliwinska et al. 2021; Temsch et al. 2021), and is sensitive enough to detect DNA changes caused by the presence or absence of one single chromosome (Pfosser et al. 1995; Galdiano et al. 2013). Hence, FCM has been successfully used to monitor the cytogenetic stability of *in vitro* propagated plants (Thiem and Sliwinska 2003; Loureiro et al. 2007; Sliwinska and Thiem 2007; Mallón et al. 2010; Galdiano et al. 2013; Corduk et al. 2018; Ulvrova et al. 2021). The basic process of plant FCM is as follows: isolation of nuclei from plant material, staining of their DNA with fluorochrome, followed by analysis of their fluorescence emission (Cires et al. 2009).

To our knowledge, no investigations have been carried out on *in vitro* propagation of any members of the genus *Tripleurospermum* till now. In this study, we have optimized a simple and rapid system for *in vitro* propagation of *T. insularum*. Besides, cytogenetic fidelity of produced plantlets was assessed with FCM and chromosome counting.

### Materials and methods

#### Plant material and achene germination

The mature achenes of *T. insularum* intended for evaluation as explants were obtained from the seed collection of the first author (accession number: Inceer 717, see in Inceer and Hayırlıoğlu-Ayaz (2014) for details of the collection information).

Initially, the achenes were thoroughly washed with tap water for 30 min, followed by treatment with 70% (v/v) ethanol (EtOH) for 30 s. After removal of the EtOH, the achenes were disinfected with 3% (v/v) sodium hypochlorite (NaOCl) for 10 min. Finally, the disinfected achenes were well rinsed with sterile distilled deionized water at least three times and each repetition lasted for 15 min, and then cultured on Murashige and Skoog (MS) (Murashige and Skoog 1962) (Duchefa Biochemie, Haarlem, The Netherlands), and Gamborg’s B-5 (BS) (Gamborg et al. 1968) (Duchefa Biochemie, Haarlem, The Netherlands) basal media, each containing 4.7 µM kinetin (KIN) (Sigma-Aldrich, St Louis, MO). Approximately 40 mL nutrient basal media were preferred in 98.5 × 59 mm glass Magenta culture vessel (Sigma-Aldrich, St Louis, MO). The pH was adjusted to 5.8 with 1 M HCl or 1 M NaOH. For preparation of most suitable germination media, 2% (w/v) sucrose (Duchefa Biochemie, Haarlem, The Netherlands) and 0.8% (w/v) phyto agar (Duchefa Biochemie, Haarlem, The Netherlands) were preferred. At the end of the 30th day, the plantlets were evaluated for germination percentage to determine which of these two main basal media were better suited to *T. insularum*.

#### Shoot multiplication

In the shoot multiplication studies, nodal segments obtained from seedling shoots after the third subculture were evaluated as an explant. MS basal medium including vitamins and containing 2% (w/v) sucrose (Duchefa Biochemie, Haarlem, The Netherlands) and 0.8% (w/v) phyto agar (Duchefa Biochemie, Haarlem, The Netherlands) was selected as the most suitable medium for shoot proliferation studies. The cytokinins 6-benzylaminopurine (6-BA, 4.4 µM), KIN (4.7 µM), 6-(y,y-dimethylallylamino)-purine (2iP, 4.9 µM), thidiazuron (TDZ, 4.5 µM) and zeatin (ZEA, 4.6 µM) were combined individually with the auxins 0.5 µM indole-3-butyric acid (IBA), indole-3-acetic acid (IAA) or α-naphthalene acetic acid (NAA). All plant growth regulators (PGRs) used in this work (supplied by Sigma-Aldrich, St Louis, MO) were sterilized with 0.22-µm filters, except for 6-BA and NAA. Filter-sterilized PGRs were added to the cooled media at approximately 40°C after autoclaving in order to maintain their structure. 6-BA and NAA were added to the culture medium before autoclaving. MS medium without any PGR was evaluated as a control. Before
autoclaving the culture media, the pH was adjusted to 5.8 with 1 M HCl or 1 M NaOH. All culture media were sterilized for 20 min at 121 °C under 1.1 kPa pressure. Culture conditions in the growth room were adjusted at 24 ± 2 °C under a 16 h photoperiod at a photosynthetic flux density of 50 μmol m⁻² s⁻¹. Cool daylight fluorescent lamps (Philips HO 49 W/840, Poland) were used for this setting. The ideal subculture times were determined as 4 weeks after the explants were transferred into the culture medium. Multiplication rates were calculated by assessing the number of shoots per explant, length of shoots, the number of leaves on each shoot and callus formation. In addition to all these, plants were evaluated in terms of plant quality with observational data such as internode length and shoot thickness.

**Flow cytometry**

Samples were processed by FCM using propidium iodide (PI)-stained nuclei to determine DNA ploidy level. Always the same individual of *T. insularum* (2n = 2x = 18, Inceer and Hayirlioglu-Ayaz 2014) was used as external standard to establish the ploidy of each treatment. For this purpose, young fresh leaves obtained from regenerated plantlets per treatment as well as young leaves of plants collected from the natural population were chopped using a razor blade in 1 mL of woody plant buffer (0.2 M Tris–HCl, 4 mM MgCl₂·6H₂O, 2 mM EDTA, Na₂H₂O, 86 mM NaCl, 10 mM K₂S₂O₅, 1% PVP-10, 1% (v/v) Triton X-100, pH 7.5; Loureiro et al. 2007) supplemented with 50 μg mL⁻¹ PI and 50 μg mL⁻¹ DNAase-free RNAse, filtered through a 30-μm mesh and stored on ice, in dark, until measurement. Samples were then analyzed using a BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA) and histograms were generated after analyses of at least 10,000 nuclei per sample (Inceer et al. 2016). All analyses were performed on the same machine with the same operator. Only histograms with coefficients of variation (CVs) of the G₁ peak of the analyzed samples below 5% were considered (Doležel et al. 2007a). Three to four plants were analyzed per treatment and each individual was repeatedly measured two times on two different days. The DNA ploidy was determined by comparing the fluorescence values of the G₁ peak positions of samples to that of the external standard (DNA peaks ratio, Doležel et al. 2007b).

**Root induction**

MS media, each individually supplemented with 2.5 μM IBA, 2.9 μM IAA and 2.7 μM NAA or without PGRs, were selected for rooting the well-proliferated and sufficiently elongated shoots (≥20 mm). After 4 weeks, the rooting parameters were calculated to evaluate the rooting success of *T. insularum*. Each rooting treatment contained a total of 16 healthy shoots and each experiment was carried out in triplicate.

**Chromosome counts**

Root tips about 1 cm long obtained from rooted shoots on MS media containing 2.5 μM IBA, 2.9 μM IAA, 2.7 μM NAA or without PGRs were used for chromosome counting. Samples were pre-treated with 2 mM 8-hydroxyquinoline solution for 6 h at room temperature. They were fixed in absolute ethanol-glacial acetic acid (3:1) for at least 24 h at 4 °C, hydrolyzed in 1 M HCl at 60 °C for 12–13 min and then rinsed in deionized water for 2–3 min. Staining was carried out in Schiff’s reagent for 2–3 h at room temperature, and squashes were made in 45% acetic acid (Inceer and Beyazoglu 2004). The preparations were then mounted in Entellan and permanent slides were prepared from at least five well-spread metaphase plates from five individuals per treatment. The best metaphase plates were photographed by means of a Leica DM 1000 microscope.

**Acclimatization**

To remove the medium residue, the roots of plantlets were washed with running tap water for a while. The rooted micro-shoots were then transferred to vermiculite supplemented with Hoagland’s nutrient solution adjusted to pH 5.8. For this process, transparent light-transmitting plastic containers of 71 × 71 mm size were preferred. The plants were subjected to the first acclimatization process for four weeks under the same culture room conditions by gradually decreasing the humidity. After that, well-developed plantlets were transplanted into a raised bed containing peat in the greenhouse. After being transplanted, the plantlets were watered and then covered with plastic film and shading net to maintain high humidity. The transplanted plantlets were cultured in the greenhouse by natural light, maintaining the temperature at 24–28 °C and the relative humidity at 80–85% during 2 weeks. After that, the plantlets were successfully transferred into multi-cell trays containing 1:1 (v/v) peat and forest soil and cultured in the greenhouse under natural light, maintaining the temperature at 24–28 °C and the relative humidity at 75–80% during 2 weeks. After being cultivated in the greenhouse, the plantlets were transferred to the botanical garden.

**Statistical analysis**

For all germination experiments, five achenes were placed into each Magenta culture vessel, and six vessels were prepared per treatment. For all shoot proliferation experiments, four nodal explants were cultured in Magenta culture vessel and six vessels were set up for each treatment. Each rooting treatment contained a total of 16 healthy shoots. To test the significance of differences in achene germination between MS and BS basal media, Student’s t-test was applied. For the data of *in vitro* propagation, Duncan’s multiple range test from one-way analysis of variance (ANOVA) was employed to detect the statistical significance of differences among the means using SPSS version 21 (SPSS Inc., Chicago, IL).

**Results**

**In vitro achene germination**

The tested sterilization method on *T. insularum* achenes was effective and achieved 100% success. No microbial
contamination was observed in the achenes transferred to the culture media. The first germination data were obtained at the end of days 8 and 10 after inoculation of the achenes in MS and B5 media, respectively. The MS medium had a better effect than B5 medium, and yielded 8% more achene germination. Fifty-eight percent germination was obtained on MS medium, compared to 50% on B5 medium. These two culture media exhibited significant differences in terms of numbers of germinated achenes (t (22)=3.804, p<.01). MS medium was therefore employed in subsequent shoot multiplication experiments with T. insularum.

**Shoot multiplication**

*In vitro* shoot proliferation from nodal explants was successfully achieved after 4 weeks. The maximum shoot number per explant (3.33) for this species was recorded with MS medium supplemented with 4.6 µM ZEA and 0.5 µM IAA (*Figure 1(a)*). TDZ applications yielded low values in terms of shoot numbers, and there were significant differences between these and other applications (F<sub>15,368</sub>=25.209, p<.001). MS medium supplemented with 4.6 µM ZEA and 0.5 µM IAA combination again reached the highest shoot elongation value with 39.68 mm. The cytokinin and auxin-free MS medium yielded the same shoot length value as MS medium added with 4.6 µM ZEA and 0.5 µM IAA (*Table 1, Figure 1(a)*). On the other hand, MS medium containing 6-BA combined with any auxin was found to be less effective than other cytokinin and auxin combinations in terms of shoot length. The lowest shoot length (13.48 mm) was obtained from MS media containing 4.4 µM 6-BA and 0.5 IAA µM (*Table 1*). Significant differences were also observed between the ZEA as well as control and other experimental objects in terms of shoot length (F<sub>15,368</sub>=393.353, p<.001).

In contrast to the mean values for shoot number and shoot length, MS medium supplemented with 4.6 µM ZEA and 0.5 µM IBA yielded the highest node number value with 10.67 nodes per explant, although it was not significantly different from the control (10.46 nodes per explant; *Table 1*). On the other hand, the statistical difference between these two applications and the others was significant (F<sub>4,95</sub>=266.429, p<.001).

In this study, evaluation of callus formation has revealed a significant response in all treatments, except for control and KIN plus IAA applications. Among all the treatments, TDZ in combination with IBA or IAA were more effective than other treatments in stimulating callus formation, resulting in 100% callus formation. ZEA and NAA (54.17%) and 2iP and IAA (50%) combinations also caused significant callus formation (F<sub>15,32</sub>=366.005, p<.001).

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**Figure 1.** Micropropagation, rooting and acclimatization of *T. insularum*. (a) Shoots induced on MS medium supplemented with 4.6 µM ZEA + 0.5 µM IAA, bar = 1 cm. (b) Rooting after 30 d on MS medium supplemented with 2.7 µM NAA, bar = 5 cm. (c) Micropropagated plants in a raised bed containing peat in greenhouse after 15 d of culture, bar = 1 cm. (d) Micropropagated plants in multi-cell trays containing peat and forest soil in greenhouse after 15 d of culture, bar = 1 cm. (e-f) Acclimatized plants in botanical garden after 15 d of culture, bars = 1 cm.
Root induction

The culture conditions during root initiation and root growth were the same as the shoot formation conditions. The obtained rooting successes differed according to the tested auxin type at the end of 4th week of culture (Table 2). Only IAA application yielded 100% rooting success for all tested plantlets \( F_{3,8} = 6.556, p < .05 \). In contrast, the highest values in terms of root number \( (F_{3,60} = 12.336, p < .001) \), root length \( (F_{3,60} = 35.748, p < .001) \) and secondary root number \( (F_{3,60} = 139.815, p < .001) \) were obtained with auxin-free and NAA-supplemented MS media.

A root length of about 20 mm under culture conditions can be considered sufficient for acclimatization studies (Figure 1(b)). Because of this, the seedlings were kept in culture conditions for 4 weeks due to the delayed initial root induction. In addition, this was necessary to determine the effect of the tested auxins in an equal period of time. Although there was little difference between the four tested media in terms of root formation times, the first root was seen on day 19 in MS medium supplemented with 2.7 µM NAA. Likewise, the highest root number (2.46 per explant) as well as secondary root number (17.63 per explant) were obtained on MS medium supplemented with 2.7 µM NAA (Table 2). On the contrary, the greatest root length detected was 59.1 mm on auxin-free. The shortest root length detected was 46.33 mm on MS medium supplemented with 2.5 µM IBA. Significant differences and similarities in terms of root length between all the tested media are shown in Table 2.

Cytogenetic stability

The G1 peak of all analyzed samples exhibited low CVs (2.31–3.71%) indicating reliability and enabling determination of DNA ploidy level \( (x) \) unequivocally (Table 3, Figure 2). According to results of FCM analysis, the DNA ploidy level of propagated plants is 2x as in mother plants \( (in vivo) \). The DNA peaks ratio (sample/standard plant) ranged from 1.08 with 4.9 µM 2iP combined with 0.5 µM IBA to 1.39 with 4.7 µM KIN combined with 0.5 µM NAA (Table 3). In addition, somatic chromosome number of all propagated plants was 2n = 2x = 18 (Table 2, Figure 3).

Ex situ collection

The rooted and well-developed plantlets were successfully acclimatized under greenhouse conditions. The plants were healthy, green and grew rapidly; some of them even formed new buds and then bloomed (Figure 1(c,d)). The survival frequency was approximately 76% for \( T. insularum \) under greenhouse conditions. After 30 d, the plants were moved outdoors to the botanical garden. After a month, they developed new leaves, and some also formed buds and then bloomed (Figure 1(e,f)). The survival frequency was recorded as approximately 74%.

Discussion

This study is the first report of a successfully developed micropropagation system for the CR insular endemic species

### Table 1. Effects on shoot multiplication of PGRs in *Tripleurospermum insularum*.

| Cytokinin (µM) | Auxin (µM) | Shoot number/explant | Shoot length/explant (mm) | Node number/explant | Callus rate (%) |
|---------------|-----------|----------------------|--------------------------|---------------------|----------------|
| Control       | KIN       | 0.0                  | 0.0                      | 0.0                 | nd             |
| 4.7           | 6-BA      | 0.0                  | 0.0                      | 0.0                 | nd             |
| 4.7           | 2iP       | 0.0                  | 0.0                      | 0.0                 | nd             |
| 4.7           | TDZ       | 0.0                  | 0.0                      | 0.0                 | nd             |
| 4.7           | ZEA       | 0.0                  | 0.0                      | 0.0                 | nd             |
| 4.7           | IBA       | 0.0                  | 0.0                      | 0.0                 | nd             |
| 4.7           | IAA       | 0.0                  | 0.0                      | 0.0                 | nd             |
| 4.7           | NAA       | 0.0                  | 0.0                      | 0.0                 | nd             |

Values (mean value ± standard deviation) within a column followed by different letters indicate significant differences according to Duncan’s multiple-range test at \( p < .05 \). Data were recorded after 4 weeks of culture and represent a total of three replicates of 24 plants per treatment.

### Table 2. Effects of different auxin types on *in vitro* rooting and chromosome number (2n) in *Tripleurospermum insularum*.

| PGRs (µM) | Rooting rate (%) | Root number per plant | Root length (mm) | Secondary root number per plant | 2n |
|-----------|------------------|-----------------------|-----------------|----------------------------------|-----|
| Control   | 91.7 ± 3.6b      | 1.65 ± 0.37c          | 59.1 ± 3.91a    | 10.8 ± 0.64d                     | 18  |
| 2.5 IBA   | 89.6 ± 3.6b      | 1.82 ± 0.39bc         | 52.91 ± 3.67b   | 17.63 ± 0.91a                    | 18  |
| 2.9 IAA   | 100 ± 0.0a       | 2.04 ± 0.33b          | 52.2 ± 2.91b    | 15.08 ± 0.97b                    | 18  |
| 2.7 NAA   | 91.7 ± 3.6b      | 2.46 ± 0.48a          | 52.1 ± 3.67b    | 17.63 ± 0.91a                    | 18  |

Values (mean value ± standard deviation) within a column followed by different letters indicate significant differences according to Duncan’s multiple-range test at \( p < .05 \). Data were recorded after 4 weeks of culture and represent a total of three replicates of 16 plants per treatment for root induction.
T. insularum using nodal segments in MS media supplemented with cytokinins (6-BA, KIN, 2iP, TDZ and ZEA) and auxins (IAA, IBA and NAA). The plants obtained in vitro via direct shoot regeneration grew well in the field and were phenotypically similar to in vivo plants (Inceer and Hayirlioglu-Ayaz 2014).

In our study, the percentage (58%) of in vitro achene germination indicates that T. insularum has a relatively low germination rate. On the other hand, its seedlings are healthy and suitable for obtaining explants. As already noted by Leyva-Peralta et al. (2019), germination rates in the achenes of the family Asteraceae are generally low because of their heterogeneous maturation. Besides, the environmental stress factors such as winter rainfall and summer drought in the Mediterranean climate can negatively affect germination in this species’ habitat.

Several combinations of cytokinins and auxins have been used to induce shoots from different types of explants in the members of the family Asteraceae (Radhika et al. 2006; Enyew and Feyissa 2019). The best condition for propagating Achillea filipendulina Lam. “Parker” (Asteraceae) was found to be MS medium supplemented with 6-BA and IAA (Evenor and Reuveni 2004). However, in this study, the use of 4.4 µM 6-BA plus 0.5 µM IAA resulted in the lowest shoot number

Table 3. Effects on DNA ploidy of PGRs in Tripleurospermum insularum.

| Cytokinin (µM) | Auxin (µM) | Number of investigated plants | DNA ploidy level (x) | DNA peaks ratio | CV (%) |
|----------------|------------|-------------------------------|----------------------|----------------|-------|
| KIN 6-BA 2iP TDZ ZEA IBA IAA NAA | | | | | |
| 4.7 0.5 4.5 4.6 | 4.4 0.5 4.5 4.6 | 3 | 2x | 1.37 | 2.61 |
| 4.7 0.5 4.5 4.6 | 4.4 0.5 4.5 4.6 | 4 | 2x | 1.17 | 3.71 |
| 4.7 0.5 4.5 4.6 | 4.4 0.5 4.5 4.6 | 5 | 2x | 1.27 | 2.43 |
| 4.7 0.5 4.5 4.6 | 4.4 0.5 4.5 4.6 | 6 | 2x | 1.29 | 2.31 |
| Control (in vitro plants without PGRs) | | | | | |

Figure 2. Representative flow cytometric histograms. (a) Peak of nuclei propagated plants on MS medium supplemented with 4.9 µM 2iP + 0.5 µM IBA. (b) Peak of nuclei propagated plants on MS medium supplemented with 4.6 µM ZEA + 0.5 µM NAA. (c) Peak of nuclei propagated plants on MS medium supplemented with 4.7 µM KIN + 0.5 µM NAA. (d) Peak of nuclei propagated plants on MS medium supplemented with 4.6 µM ZEA + 0.5 µM IBA. (e) Peak of nuclei propagated plants on MS medium supplemented with 4.9 µM 2iP + 0.5 µM IAA. (f) Peak of nuclei control (in vitro plants without PGRs).
and length, indicating that they are not as effective in shoot regeneration for *T. insularum*. Among the various PGRs, the highest multiplication rate was obtained in the presence of 4.6 µM ZEA and 0.5 µM IAA. These results are in agreement with the previous report for *Helichrysum italicum* (Roth) G.Don (Asteraceae) (Giovannini et al. 2003).

Callus formation was observed with all PGR combinations, except KIN plus IAA. Percentage callus formation was very high in MS medium supplemented with 4.5 µM TDZ and 0.5 µM IBA as well as 4.5 µM TDZ and 0.5 µM IAA. Additionally, the use of 4.5 µM TDZ in combination with 0.5 µM IBA resulted in the lowest shoot number and length, indicating that this treatment was not as effective in shoot multiplication for this species. Similar results were reported from other species of Asteraceae such as *Gerbera jamesonii* H.Bolus ex Hook. (Chakrabarty and Datta 2008) and *Stevia rebaudiana* Bertoni (Zayova et al. 2013).

The auxins IAA, IBA and NAA have been used in different concentrations for rooting of species from the Asteraceae family (Trejgell et al. 2010, 2018; Cardoso and Teixeira da Silva 2013; Shinde et al. 2017). On the other hand, roots can also develop on MS medium lacking auxin (Opabode et al. 2017). It has been reported that auxins increase rooting in *Saussurea obvallata* (DC.) Edgew. (Joshi and Dhar 2003). The present results revealed that IAA and NAA were more effective than IBA in the induction of roots in regenerated shoots of *T. insularum*. Additionally, as already noticed by Opabode et al. (2017), the incidence of root formation on auxin-free medium may be due to the presence of endogenous auxin in regenerated shoots. Similar findings were also found in other members of Asteraceae such as *Inula verbascifolia* (Willd.) Hausskn. on MS medium plus 2.9 µM IAA, and *Gundelia tournefortii* L. on MS medium plus 2.7 µM NAA, respectively (Owies et al. 2004; Perica et al. 2008).

The chromosome number of *T. insularum* was reported by Inceer and Hayirlioglu-Ayaz (2014) as 2n = 2x = 18. The results obtained from FCM analysis and chromosome counting revealed that all of the propagated plants maintained the diploid condition as in mother plants. This finding indicated that the *in vitro* propagation protocol apparently did not induce genome multiplication in the propagated plants. However, we cannot exclude presence of aneuploids regarding the limits of FCM measurements performed in this study (external standardization).

The present results showed that there were slight differences in the DNA peaks ratio despite the stable DNA ploidy level (2x). As noticed by Loureiro et al. (2021), staining inhibition associated with the presence of secondary metabolites, such as phenolic compounds may lead to variation in nuclear fluorescence intensity. The differences in the DNA peaks ratio may be due to the different concentrations of phenolic compounds between *in vivo* external standard and *in vitro* propagated plants.

**Conclusion**

In conclusion, this study is the first report of a successfully developed *in vitro* propagation protocol for *ex situ* conservation of *T. insularum*. The present results also confirm that the proposed *in vitro* propagation protocol guarantees the cytogenetic fidelity of regenerated plantlets of *T. insularum*. The propagated plantlets are well-acclimatized to the *ex vitro*
conditions. Hence, this protocol may be also used in future conservation programs, such as in situ activities on population restoration and cryopreservation of this species as well as other threatened endemic species of *Tripleurospermum*.

**Acknowledgements**

We would like to thank Betul Ergin for technical assistance; greenhouse personnel for ex vitro studies in Faculty of Forestry at the Karadeniz Technical University. The authors also express their thanks to anonymous reviewers and subject editor for their comments and suggestions which helped to improve the manuscript considerably.

**Disclosure statement**

The authors declare that no conflict interests.

**Funding**

This work was supported by the Scientific and Technological Research Council of Turkey [TUBITAK project no (grant numbers) 117Z588 and 106T162].

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