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In vitro propagation, antioxidant and antimicrobial activities of *Tripleurospermum baytopianum* (Asteraceae): a threatened endemic species in Türkiye

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ABSTRACT

In this study, a rapid and efficient *in vitro* propagation protocol has been established for threatened endemic species *Tripleurospermum baytopianum* (Asteraceae) growing naturally in Türkiye in Murashige and Skoog basal medium (MS) using nodal explants. The achenes evaluated as initiation material reached the highest germination success of 76.7 % in MS medium supplemented with 1.0 mg L^{-1} gibberellic acid (GA_3). MS medium containing 1.0 mg L^{-1} 6-BA and 0.1 mg L^{-1} IBA was found to be the best medium to stimulate and sustain the new shoot formation (8.3 shoot number per explant). This medium was also more effective in terms of callus formation, fresh and dry weight parameters per explant with 87.8 %, 1.74 g and 0.111 g, respectively. MS basal medium enriched with 0.5 mg L^{-1} 2iP plus 0.1 mg L^{-1} NAA achieved 74.7 mm shoot length per explant was quite remarkable. Rooting success was achieved between 86.7 % and 100 % in all rooting media. The acclimatization process of the plantlets was carried out successfully with 75 % survival rates in botanical garden. Cytogenetic analysis revealed that propagated plants have the same chromosome number ($2n = 2x = 18$) with their mother plants in natural population, indicating genetic stability. Screening of the antioxidant (DPPH scavenging) and antimicrobial (disc diffusion) activities of ethanolic extracts of *in vitro* propagated plants were also carried out and compared with that of the wild-grown plants (mother plants). The biochemical tests showed that *in vitro* propagated plants and the mother plants have almost the same antioxidant activities, indicating metabolic stability. On the other hand, although statistically the highest antimicrobial activity was in sample 20 and sample 1 (taken from nature), it can be said that the high amount of cytokinin relatively increases the antimicrobial activity. The study signifies a notable *in vitro* production technique for *T. baytopianum* and provides an important comparison method of the antioxidant and antimicrobial potentials of *in vitro* derived shoots and mother plants of threatened endemic *T. baytopianum*.

1. Introduction

Tripleurospermum Sch.Bip. is a typically Mediterranean genus of the tribe Anthemideae of the family Asteraceae with *ca.* 40 species that are mainly distributed in Europe, temperate Asia, North America, and North Africa (Oberprieler et al., 2007). It is particularly abundant and diverse in Türkiye, its main center of diversity with *ca.* 33 taxa in Türkiye, of which 17 are endemic (Cuce and Inceer, 2024). However, some of the endemic taxa are face to extinction risk due to several anthropogenic pressures such as chemical pollution, habitat fragmentation, grazing, and all kinds of activities within the scope of nature tourism in this country (Ekim et al., 2000).

The members of the *Tripleurospermum* genus have pharmaceutical values such as anti-inflammatory, antiseptic, antifungal, antibacterial, antiulcer, and antioxidant potentials (Colak et al., 2017; Erdogan et al., 2013; Zeljkovic et al., 2015). 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 (Mohammadi et al., 2016; Nadiroglu et al., 2019).

Tripleurospermum baytopianum E.Hossain is an endemic species for Türkiye (Enayet Hossain, 1975). It mainly grows on slopes, meadows, roadsides, and *Pinus brutia* forest (Enayet Hossain, 1975; Inceer

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and Ozcan, 2021; Inceer et al., 2018). It is a diploid species with $2n = 2x = 18$ chromosomes (Inceer et al., 2018). *Tripleurospermum baytopianum* is biennial or perennial herb. Stems are solitary or numerous, unbranched or rarely branched. Leaves are ± two-pinnatisect. Capitula are usually solitary, terminal, rarely two on each branch, radiate. Ray flowers are white, disc flowers are yellow with five deltoid lobes, and corolla lobes of disc flowers are glandular at the tips. Achenes are linear-oblong oblong, mucilaginous, pale brown at maturity (Enayet Hossain, 1975).

According to Ekim et al. (2000), *T. baytopianum* is classified within the endangered (EN) category of IUCN (The International Union for Conservation of Nature). The IUCN Red List extinction data of *T. baytopianum* are associated with low competitive ability and several anthropogenic pressures, such as habitat fragmentation, overgrazing, trampling and soil erosion. These anthropogenic pressures can cause a decrease in population size and density of this species. 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 techniques have often been successfully utilized in propagation of rare and endangered species of plants as in some threatened endemic species of the genus *Tripleurospermum* (Cuce et al., 2022; Cuce and Inceer, 2024; Inceer et al., 2022). On the other hand, the genetic fidelity of propagated plants is required to test the success of *in vitro* propagation protocols for conservation programs due to somaclonal variations associated with stress conditions during tissue culture (Bairu et al., 2011; Slazak et al., 2015; Ulvrova et al., 2021). The cytogenetic fidelity of the propagated plants could be practically tested by cytogenetic methods such as chromosome counting and flow cytometric (FCM) analysis (Cuce et al., 2022; Inceer et al., 2022; Mahanta et al., 2023) or 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 (Corduk et al., 2018; Park et al., 2020; Ulvrova et al., 2021).

In addition to the importance of conservation of the species of *in vitro* propagation, it is also used for the improvement of valuable secondary metabolites (Chandran et al., 2020; Das et al., 2023). Under *in vitro* conditions, the plant cell produced a large amount of biomass which has gained viable production of secondary metabolites that can contribute to their antioxidant activity (Das et al., 2023). It is well known that many plants species have a broad range of bioactive compounds that serve a variety of biological purposes. Because they neutralize reactive oxygen species (ROS) and reduce the damage they cause to cellular structures, antioxidants are crucial to this abundant reserve. The antioxidant capability of plants has attracted a lot of attention in the search for natural solutions to fight oxidative stress and related health problems such as cancer, neurological conditions, and cardiovascular diseases. These results from an imbalance between the generation of ROS and the cellular antioxidant defense mechanism (Bhattacharya, 2015; Singh, 2016). As a result, the search for potent antioxidants has become central to preventative research and efforts to develop treatment plans for diseases associated with oxidative stress. Plant growth regulators (PGRs) used in the regulation of plant growth are known to play an important role in the synthesis of secondary metabolites that contribute to antimicrobial activity (Bhattacharyya et al., 2023). The antimicrobial secondary metabolites produced are important in plant adaptation to the environment and in the fight against soil-borne pathogens (Kasem, 2017). Therefore, DPPH scavenging activity method was preferred for the determination of antioxidant activity and Kirby-Bauer disk diffusion method was preferred for the determination of antimicrobial activity.

To the best of our knowledge, *in vitro* propagation, antioxidant, and antimicrobial activities of threatened endemic *T. baytopianum* are not reported in the literature at present. The present study aimed to develop an efficient and effective *in vitro* propagation protocol for *T. baytopianum* from nodal segments. Based on the fact that plant *in vitro* propagation sometimes causes somaclonal variation (Inceer et al., 2022), we tested the genetic fidelity of the regenerants using chromosome counting. Finally, the antioxidant and antimicrobial activities of shoots of regenerated (*in vitro*) and mother plants (*in vivo*) were assessed.

2. Material and methods

2.1. Plant material and achene germination

The dark brownish mature achenes obtained from five individuals in the natural population were evaluated as the initial material provided from collection of *T. baytopianum* (Türkiye:A1(E) Canakkale, Koru Mountain, near *Pinus brutia* forest, 350 m a.s.l.,11.v.2007, Inceer 333, KTUB). Sterilized achenes were aseptically cultivated in Murashige and Skoog basal medium including vitamins (MS) (Murashige and Skoog, 1962) (Duchefa Biochemie, Haarlem, The Netherlands) both supplemented with gibberellic acid (1.0 mg L^{-1} GA_3, Sigma-Aldrich, St Louis, MO) and without any plant growth regulators (PGRs-free or control). The ideal achene germination period has been determined as 4 wk. At the end of this time, germination percentages of achenes were calculated for both treatments. Obtained plantlets were subcultured at least three times after germination until sufficient nodal explants were obtained for shoot multiplication studies.

2.2. Experimental

2.2.1. Sterilization

T. baytopianum dark brownish mature achenes were initially washed with tap water on a magnetic stirrer (Heidolph Instruments, Germany) for about 1 h. After the tap water residues were completely removed, the achenes were quickly agitated (30 s) with freshly prepared 70 % (v/v) ethanol (EtOH) as a second pretreatment and the ethanol residues were completely removed from the environment. In the final process, the achenes were subjected to thorough sensitive surface sterilization in 20 % commercial bleach (Domestos) on a magnetic stirrer (500 rpm) for about 10 min. To completely remove the all above-mentioned sterilization agent residues, the deeply sterilized achenes were successively rinsed with double distilled water for 4 times (each repetition at least 5 min) in a sterile laminar airflow (SafeFAST premium 212, Ferrara, Italy) in the following stage. Subsequently, these achenes were directly inoculated to the tissue culture vessels (Magenta™ glass containers, Sigma-Aldrich, St Louis, MO) containing nutrient MS basal medium (about 40 mL). Inoculated culture media were maintained in the growth room until the end of the germination process

2.2.2. Shoot proliferation

Nodal explants from excised from four months old *in vitro* produced plantlets of *T. baytopianum* were preferred as explants. MS nutrient basal medium including 2.0 mg L^{-1} glycine, 100.0 mg L^{-1} myo-inositol, 0.5 mg L^{-1} nicotinic acid, 0.5 mg L^{-1} pyridoxine HCl and 0.1 mg L^{-1} thiamine HCl vitamins were used for shoot multiplication studies. Aseptic five explants were cultivated in a 98.5 × 59 mm glass Magenta™ culture vessel (Sigma-Aldrich, St Louis, MO) containing about 40 mL MS solid medium with 2 % (*w/v*) sucrose (Duchefa Biochemie, Haarlem, The Netherlands) and 0.8 % (*w/v*) plant agar (Duchefa Biochemie, Haarlem, The Netherlands). The pH of the media was adjusted to 5.8 before autoclaving using 1 M HCl or 1 M NaOH. Media individually supplemented with 0.25, 0.5 and 1.0 mg L^{-1} concentrations of 6-benzyladenine (6-BA), kinetin (KIN), 6-(y,y-dimethylallylamino)-purine (2iP) and in combination with 0.1 mg L^{-1} indole-3-butyric acid (IBA), or α-naphtaleneacetic acid (NAA), for high frequency shoot multiplication experiments. All PGRs (Sigma Sigma-Aldrich, St Louis, MO) were sterilized with cellulosic 0.22 μm Millex-

GS filters (Merck Millipore, Tullagreen, Carrigtwohill, Co. Cork, Ireland) and were added to the culture media after autoclaving except for 6-BA and NAA. In the climate room conditions, incubation was performed at 24 ± 2 °C and a 16/8 h photoperiod at an irradiance of 50 μmol m^{-2} s^{-1} with cool white daylight fluorescent light lamps (Lumilux, HE 21W/840, Osram, Italy) and all cultures were incubated in these conditions throughout the subculture period. Data for the mean number of shoots, shoot lengths, node number, callus formation, dry weight, and fresh weight per explant were calculated for shoot multiplication efficiency at the end of the four wk.

2.2.3. Cytogenetic analysis

Well-developed and active elongation phase root tips obtained from the *in vitro* derived shoots were used for chromosome counts. For this purpose, root tips were pre-treated with 0.05% colchicine solution at room temperature, fixed in ethanol-acetic acid (3:1) for at least 24 h at 4°C, hydrolysed in 1 M HCl at 60°C for 10 min and then rinsed in tap water for a minimum of 2-3 min (Inceer and Beyazoglu, 2004). Staining was carried out in 1 % aqueous lacto-propionic orcein for 12–18 h at room temperature, squashes were made in 45 % acetic acid, and the preparations were mounted in Entellan. At least five well-spread metaphase plates from five individuals (plantlets) were evaluated for chromosome counting.

2.2.4. Root induction

Well developed *in vitro* derived shoots (approximately ≥ 2 cm) were excised carefully and transferred to MS basal media individually enriched with different concentrations of IBA or NAA (each one 0.25, 0.5 and 1.0 mg L^{-1}). MS medium without any auxin was tested as control for an effective root induction. After 4 wk of the culture process, % rooting success, number of roots, length of roots, and number of secondary roots (the lateral roots on the longest root) per explant data were recorded.

2.2.5. Acclimatization

Plantlets with well-rooted and single shoots were removed gently from the Magenta™ culture vessels and washed sensitively under running tap water for removal of adhered culture media. To increase acclimatization ability, the root length of the shoots was adjusted to approximately 40 mm. In the first stage, enough sufficiently long and rooted plantlets were kept in plastic containers containing only vermiculite supplemented with Hoagland's nutrient solution (pH 5.8) for 4 wk for an effective acclimatization process in climate room conditions. The second stage of acclimatization was carried out in greenhouse conditions containing (100 % peat, 80 % humidity, and 25 ± 2 ° C temperatures). The survival frequencies of plantlets were determined after both acclimatization stages. Following these stages, healthy surviving plantlets were transferred to a botanical garden containing 3:1 (v/v) peat and perlite to strengthen the plantlets.

2.3. Chemicals and equipments

All chemicals and solvents were supplied from Sigma-Aldrich or TCI and used without additional purification. A spectrophotometer; BioTek/Epoch 2 Microplate reader, was used to measure the absorbance values at a certain wavelength. A rotary evaporator (Heidolp-2) attached to a vacuum pump (Heidolph Rotavac Vario) was used in order to remove the solvents and prepare the extracts.

2.4. Extract preparation

Explants obtained after achene germination were subcultured three times every 4 wk. For the extraction process, some shoots were collected from the culture media after the 4th subculture. This procedure was carried out just before the process of rooting the shoots in the culture medium. 80 ml of absolute EtOH were mixed with 4 g of the young shoots obtained from different PGRs treatments in MS media as well as mother plants obtained from natural population. The maceration samples, which are totally 20, were left in a dark place at room temperature for two days and then transferred to the fridge for an additional three days. Subsequently, the extract solutions were filtered and evaporated under reduced pressure. All extracts were stored at −20 °C until use. Table 1 provides details on the cultivation conditions and extracts yields for the samples of *T. baytopianum*.

Table 1
Tripleurospermum baytopianum samples' cultivation conditions and extract yields (%).

Samples	Cytokinin (mg L^{-1})	Auxin (mg L^{-1})	Extract yield (%)
1 (*in vivo* plants from natural population)	Natural	Natural	5.10
2 (*in vitro* plants without PGRs)	Control	Control	7.27
3	0.25 6-BA	0.1 IBA	5.18
4	0.5 6-BA	0.1 IBA	7.26
5	1.0 6-BA	0.1 IBA	7.23
6	0.25 KIN	0.1 IBA	7.22
7	0.5 KIN	0.1 IBA	5.10
8	1.0 KIN	0.1 IBA	5.33
9	0.25 2iP	0.1 IBA	5.53
10	0.5 2iP	0.1 IBA	5.59
11	1.0 2iP	0.1 IBA	4.54
12	0.25 6-BA	0.1 NAA	7.23
13	0.5 6-BA	0.1 NAA	5.70
14	1.0 6-BA	0.1 NAA	7.45
15	0.25 KIN	0.1 NAA	6.54
16	0.5 KIN	0.1 NAA	5.34
17	1.0 KIN	0.1 NAA	5.86
18	0.25 2iP	0.1 NAA	7.89
19	0.5 2iP	0.1 NAA	6.44
20	1.0 2iP	0.1 NAA	6.89

6-BA: 6-benzyladenine; KIN; Kinetin; 2iP: 6-(y,y-dimethylallylamino)-purine; NAA: α-naphthalene acetic acid; IBA: indole-3-butyric acid.

2.5. Antioxidant activity determination

To evaluate the antioxidant properties of the samples, we employed the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity assay. Initially, a pre-trial was conducted to establish the working concentrations of the samples, resulting in a range of 100, 150, 200, 250, and 300 μg extract/ml ethanol. Subsequently, each sample and the standards butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) were prepared in ethanol according to the determined concentrations. For each of the 5 different concentrations, 500 μl of the sample was taken, followed by the addition of 3 ml of ethanol. The samples were then mixed with 300 μl of 0.25 mM DPPH ethanolic solution, and the resulting solutions were allowed to stand at room temperature in a dark place for 70 min. The absorbance values of the samples were measured at a wavelength of 517 nm using a microplate reader spectrophotometer. DPPH scavenging activity was calculated according to the following equation; where A is the absorbance of the samples, A_0 is the absorbance of the control solution containing all test components except the sample (Brand-Williams et al., 1995).

$$\text{DPPHscavengingactivity}\ (\%) = [(Ao - A)/A_0] \times 100$$

2.6. Antimicrobial activity

The antimicrobial activities of the young shoots obtained from different PGRs treatments in MS media as well as mother plants obtained from natural population were determined by the Kirby-Bauer disk diffusion test (Hudzicki, 2009). For this purpose, three Gram-positive bacteria (*Bacillus cereus* ATCC®10876, *Staphylococcus*

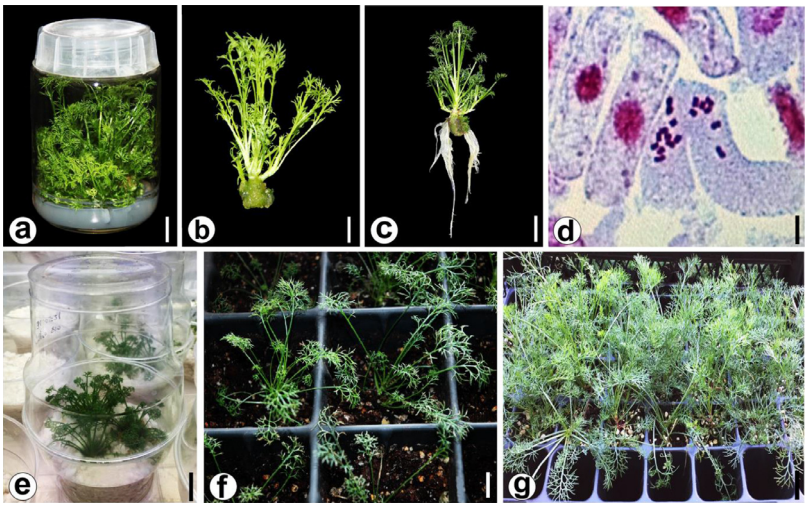

Fig 1. *In vitro* propagation *Tripleurospermum baytopianum*. (a) The most effective shoot multiplication medium (1.0/0.1 mg L^{-1} 6-BA/IBA). (b) The highest callus formation of *T. baytopianum* (1.0/0.1 mg L^{-1} 6-BA/IBA). (c) 0.5 mg L^{-1} IBA on *in vitro* rooting of *T. baytopianum* from shoot-bud culture-derived seedlings. (d) chromosomes of *in vitro* plants on Murashige and Skoog medium supplemented with 0.5 mg L^{-1} NAA. (e) Acclimatized plants in climate room conditions. (f) Acclimatized plants in greenhouse conditions. (g) Acclimatized plants in botanical garden. Bars = *a* 13 mm; b 15 mm; c 12 mm; d 10 μm.; e 22 mm; f 25 mm; g 30 mm.

aureus subsp. *aureus* ATCC 6538, *Micrococcus luteus* NRRL B-1018) and three Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC®27853, *Salmonella enterica* subsp. *enterica* ATCC14028, *Klebsiella pneumoniae* subsp. *pneumoniae* ATCC®13883) and one yeast strain (*Saccharomyces cerevisiae* ATCC®MYA-796TM) obtained from culture collections were used. The experiments were carried out in triplicate in 90 mm petri dishes with 20 mL Mueller-Hinton Agar (MHA) medium at 37°C. The antibiotic gentamicin was chosen as the standard of comparison. Gentamicin 0.5 mg mL^{-1} (for bacteria), Nystatin 100 U μg (for yeast), and 70% ethanol were used as the control groups. Inhibition zone diameters were measured with a digital caliper.

2.7. Statistical analysis

All *in vitro* production stages were performed according to a completely random factorial design as triplicates namely 10 achenes × 5 Magenta™ for germination studies, 5 nodal explants × 6 Magenta™ for shoot proliferation studies, and 4 microshoots × 6 Magenta™ for root induction studies. The means of the collected continuous data of *in vitro* production studies and antioxidant activity (DPPH scavenging activity (%)) were analyzed using the statistical package SPSS 26.0 one-way ANOVA (SPSS Inc, Chicago, IL, USA) for assaying the significant differences using Duncan's and Tukey's Multiple Range Tests, respectively. Because the arithmetic means data of the antimicrobial activity test results did not show a normal distribution, non-parametric Kruskal–Wallis analysis, and tests were preferred. In addition, pairwise comparison analysis was tested from non-parametric tests to understand which two groups had the difference. The *p* values below 0.05 were considered statistically significant for all experiments with the standard deviation (SD) by taking the arithmetic mean of the trials.

3. Results

3.1. Achene germination

The test with commercial bleach (20 %) showed 100 % positive effect and no visible contamination was determined on deeply sterilized achenes. This result was very beneficial for effective germination and subsequent *in vitro* production process. The properly sterilized achenes started germination within 6th d after inoculation in MS basal media containing 1.0 mg L^{-1} GA_3 and PGRs-free and reached the maximum percent germination after 4 wk. Based on the results of the achene germination, MS basal media augmented with 1.0 mg L^{-1} GA_3 gave 76.7 % germination success at the end of the 4 wk. This ratio was recorded as 71.1 % in the control group. The difference between these two applications was statistically significant ($p < 0.05$).

3.2. Shoot proliferation

Of the three cytokinins (6-BA, KIN and 2iP) concentrations (0.25, 0.5 and 1.0 mg L^{-1}) individually combined with IBA or NAA (0.1 mg L^{-1}), 1.0 mg L^{-1} 6-BA plus 0.1 mg L^{-1} IBA was most effective in terms of the shoot number (8.3 fold per explant). Compared to 6-BA plus tested both auxin, the shoot number increase was less effective on other treatments and this difference was statistically significant (Fig. 1a; $p < 0.05$). The lowest result in terms of the shoot number was calculated in the control. However, the incorporation of cytokinins combined with auxins in the MS culture medium improved the new shoot formations in *T. baytopianum* (Table 2). In general, 2iP treatments were determined most effective to further increase the mean shoot length per explant compared to 6-BA or KIN. MS medium augmented with 2iP (0.5 mg L^{-1}) along with NAA (0.1 mg L^{-1}) reached the highest shoot length with 74.7 mm per explant. Together with this, 2iP plus IBA applications also gave significant results in terms of the *in vitro* shoot length of *T. baytopianum* according to the control (Table 2; $p < 0.05$). Except for shoot length, 6-BA treatments showed a strong performance on all other tested parameters depending on the type of combined auxin. Namely, while the highest mean number of nodes (13.1) was obtained from the 0.5 mg L^{-1} 6-BA along with 0.1 mg L^{-1} NAA applications, 1.0 mg L^{-1} 6-BA plus 0.1 mg L^{-1} IBA reached the highest values in terms of the callus formation rate (87.8 %; Fig. 1b), fresh (1.74 g) and dry weight (0.111 g) parameters per explant. Statistical analyses revealed notable differences between all above-mentioned values and these values were presented in Table 2 ($p < 0.05$). Considering the biomass increase *in vitro* culture conditions, control treatments were found to be quite ineffective compared to the tested cytokinin/auxin concentrations in the MS medium.

3.3. Root induction

The uniformly *in vitro* derived shoots of *T. baytopianum* produced roots testing both various concentrations of IBA and NAA. The desired

Table 2
Effects of different cytokinin in the presence of IBA and NAA (0.1 mg L^{-1}) combination and control group on the shoot proliferation of *Tripleurospermum baytopianum*.

Cytokinin (mg L^{-1})				Auxin (mg L^{-1})							
Control	6-BA	KIN	2iP	IBA	NAA	Shoot number/ Explant	Shoot length/ Explant (mm)	Node number/ Explant	Callus formation (%)	Fresh weight/ Explant (g)	Dry weight/ Explant (g)
0.0	0.0	0.0	0.0	0.0	0.0	2.5 ± 0.51k	57.2 ± 3.65i	11.8 ± 1.61cde	00.0 ± 0.00l	0.24 ± 0.044j	0.024 ± 0.004k
	0.25			0.1		6.5 ± 0.68d	41.2 ± 3.04n	12.6 ± 1.30ab	38.9 ± 1.g92g	1.43 ± 0.073b	0.087 ± 0.005e
	0.5			0.1		7.5 ± 0.57b	52.1 ± 3.30k	11.8 ± 1.42cde	44.4 ± 1.93f	1.21 ± 0.046d	0.091 ± 0.007d
	1.0			0.1		8.3 ± 0.78a	50.1 ± 2.97lm	12.3 ± 1.36bcd	87.8 ± 3.85a	1.74 ± 0.046a	0.111 ± 0.013a
		0.25		0.1		4.7 ± 0.48h	69.5 ± 2.88e	11.7 ± 1.58defg	31.1 ± 1.92hi	0.83 ± 0.055fg	0.075 ± 0.006f
		0.5		0.1		5.5 ± 0.51f	70.1 ± 5.28de	11.0 ± 1.36fgh	21.1 ± 1.92j	0.84 ± 0.054fg	0.084 ± 0.008e
		1.0		0.1		5.0 ± 0.g62g	66.3 ± 3.25f	10.9 ± 1.02gh	15.0 ± 0.0k	0.97 ± 0.121e	0.091 ± 0.004d
			0.25	0.1		3.6 ± 0.50i	71.3 ± 3.59cde	10.7 ± 1.09h	34.5 ± 3.85h	0.48 ± 0.052i	0.048 ± 0.004j
			0.5	0.1		3.9 ± 0.43i	73.2 ± 3.44ab	12.3 ± 1.14bcd	28.9 ± 1.92i	0.73 ± 0.064h	0.069 ± 0.006h
			1.0	0.1		3.7 ± 0.52i	70.8 ± 2.73de	11.6 ± 1.10defg	32.2 ± 1.92hi	0.72 ± 0.057h	0.071 ± 0.006gh
	0.25				0.1	7.5 ± 0.73b	49.3 ± 3.61m	11.9 ± 1.34bcde	71.1 ± 1.92c	1.73 ± 0.167a	0.100 ± 0.009b
	0.5				0.1	7.5 ± 0.68b	52.6 ± 2.83k	13.1 ± 1.25a	76.7 ± 3.34	1.31 ± 0.076c	0.086 ± 0.005e
	1.0				0.1	7.1 ± 0.85c	51.8 ± 2.95kl	10.6 ± 1.19h	76.7 ± 3.34b	1.42 ± 0.058b	0.095 ± 0.004c
		0.25			0.1	5.5 ± 0.51f	59.9 ± 3.77h	11.9 ± 1.34bcde	42.2 ± 3.85fg	0.80 ± 0.g064g	0.074 ± 0.005e
		0.5			0.1	4.5 ± 0.51h	61.7 ± 2.g71g	11.7 ± 1.26defg	41.1 ± 1.92fg	0.81 ± 0.094fg	0.076 ± 0.005f
		1.0			0.1	5.9 ± 0.68e	55.0 ± 3.05j	11.2 ± 1.27efgh	54.4 ± 1.93e	0.85 ± 0.057f	0.064 ± 0.006i
			0.25		0.1	3.8 ± 0.43i	71.9 ± 3.12bcd	12.5 ± 1.38abc	40.0 ± 0.g00g	0.82 ± 0.041fg	0.073 ± 0.004fg
			0.5		0.1	3.6 ± 0.50i	74.7 ± 4.26a	12.3 ± 1.30bcd	54.5 ± 3.85e	0.70 ± 0.055h	0.063 ± 0.009i
			1.0		0.1	2.8 ± 0.48j	73.0 ± 3.17abc	12.3 ± 1.67bcd	60.0 ± 0.00d	0.71 ± 0.041h	0.070 ± 0.007gh

Data were recorded four weeks after the culture and represent a total of three replicates of 30 plants per treatment on MS for shoot proliferation. Values (mean value ± standard deviation) 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. KIN = Kinetin, 2iP = N^6 -(2-isopentenyl)adenine, 6-BA = 6-benzyladenine, IBA = Indole-3-butyric acid, NAA = α-naphthalene acetic acid.

Table 3
The effects of two different auxin types on *in vitro* rooting and chromosome number (2*n*) in *Tripleurospermum baytopianum*.

Auxin (mg L^{-1})						
Control	IBA	NAA	Rooting (%)	Root Number (No/Plant)	Root Length (mm)	Secondary Root Number (No/Plant)	2*n*
0.0			86.7 ± 2.31b	3.8 ± 0.48d	100.8 ± 6.57a	10.9 ± 1.14a	18
	0.25		100 ± 0.00a	8.5 ± 0.78b	60.2 ± 3.47d	10.7 ± 1.24a	18
	0.5		98.7 ± 2.31a	10.8 ± 1.19a	55.0 ± 2.55f	8.7 ± 1.09b	18
	1.0		100 ± 0.00a	8.4 ± 0.88b	57.8 ± 3.02e	8.8 ± 1.26b	18
		0.25	100 ± 0.00a	7.0 ± 0.83c	83.5 ± 4.30b	6.8 ± 0.88c	18
		0.5	100 ± 0.00a	7.4 ± 0.76c	74.2 ± 3.95c	7.0 ± 0.81c	18
		1.0	97.3 ± 2.31a	8.9 ± 0.80b	55.6 ± 3.02ef	4.4 ± 0.50d	18

Data were recorded on the four weeks after the culture and represent a total of three replicates of 24 plants per treatment for root induction. Values (mean value ± standard deviation) 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, NAA = α-naphthalene acetic acid.

rooting success was achieved in all tested media and no visible contamination or plant loss was observed. The rooting frequency of all treatments varied from a minimum of 86.7 % to a maximum of 100 %. The rooting percentages were achieved on MS media individually containing 0.25 or 1.0 mg L^{-1} IBA and 0.25 or 0.5 mg L^{-1} NAA with 100 %. However, only the control group (86.7 %) was statistically weaker than the other treatments in terms of rooting percentage (Table 3; $p < 0.05$). Among the different concentrations of these two auxins, IBA (0.5 mg L^{-1}) reached the highest mean root number with 10.8 roots per shoot. The weakest effect in terms of this parameter was calculated in the control with 3.8 roots per shoot. The effect of tested NAA concentrations was slightly less effective in terms of the root number according to IBA. Between all the concentrations of NAA, the minimum (7.0) and maximum (8.9) mean root number per shoot was carried out in MS media augmented with 0.25 and 0.5 mg L^{-1} NAA. Although the rooting percentage of MS PGR-free media was slightly lower than auxin-applied MS media, it was more effective in terms of root length and secondary root number. The highest root length and secondary root number were calculated as 100.8 mm and 10.9 per shoot, respectively in control (Fig. 1c). MS medium supplemented with various concentrations of two different auxins were statistically less powerful in terms of the above mentioned rooting parameters according to the control (Table 3; $p < 0.05$).

3.4. Chromosome number

The chromosome number of *T. baytopianum* was determined by Inceer at al. (2018) as $2n = 2x = 18$, previously. Chromosome counting showed that chemicals and culture conditions used *in vitro* did not cause genome folding and all plantlets produced *in vitro* maintained a diploid chromosome status (i.e, $2n = 2x = 18$) like naturally growing plants (mother plants) (Fig. 1d; Table 3).

3.5. Acclimatization and ex situ collection

Around 200 individuals of the *in vitro* produced plantlets of *T. baytopianum* were transferred from MS culture condition to plastic pots containing 100 % perlite under climate room conditions. Perlite was enriched with Hoagland solution (pH 5.8) to increase the acclimatization ability of rooting plantlets. At this stage, climate room conditions were kept the same as those determined in the shoot multiplication

Table 4
DPPH scavenging activity (%) of *T. baytopianum* samples (1-20).

DPPH activity (%)*	100 μg/ml	150 μg/ml	200 μg/ml	250 μg/ml	300 μg/ml	SC_{50} μg/ml
1	$23.66 \pm 0.83^{f,g,h,i,j}$	$32.55 \pm 2.48^{g,h,i}$	$50.66 \pm 2.40^{d,e,f,g}$	$64.19 \pm 4.58^{b,c}$	$71.28 \pm 0.89^{d,e,f,g}$	4.18
2	38.21 ± 2.24^{c}	$41.98 \pm 3.27^{c,d,e}$	$52.26 \pm 1.89^{d,e,f}$	$62.55 \pm 1.43^{c,d}$	$74.31 \pm 2.47^{c,d,e}$	3.88
3	39.93 ± 3.12^{c}	47.32 ± 1.43^{c}	$64.61 \pm 2.57^{c,d}$	70.78 ± 2.85^{b}	$76.13 \pm 0.71^{b,c,d}$	3.51
4	$34.16 \pm 2.57^{c,d,e}$	$43.23 \pm 1.35^{c,d}$	$51.03 \pm 2.57^{d,e,f,g}$	71.61 ± 1.24^{b}	$79.84 \pm 3.11^{a,b,c}$	3.73
5	$28.43 \pm 2.17^{e,f,g}$	$32.51 \pm 2.57^{g,h,i}$	$47.36 \pm 3.68^{e,f,g,h}$	$51.05 \pm 3.87^{g,h,i,j}$	$54.81 \pm 0.65^{k,l}$	4.90
6	16.14 ± 3.17^{k}	$40.51 \pm 1.35^{d,e,f}$	$41.91 \pm 3.34^{h,i}$	$46.91 \pm 1.24^{h,i,j,k}$	$58.03 \pm 5.66^{i,j,k,l}$	4.97
7	$17.28 \pm 2.14^{j,k}$	23.87 ± 1.89^{j}	$55.42 \pm 1.37^{d,e}$	$55.14 \pm 1.42^{d,e,f,g}$	$69.93 \pm 2.68^{d,e,f,g,h}$	4.42
8	$33.73 \pm 1.77^{c,d,e}$	$40.0 \pm 1.18^{d,e,f}$	$50.98 \pm 0.68^{d,e,f,g}$	$58.82 \pm 3.53^{c,d,e,f}$	$65.88 \pm 2.36^{f,g,h,i}$	4.21
9	$25.49 \pm 0.68^{f,g,h}$	$41.96 \pm 1.36^{c,d,e}$	$51.37 \pm 1.36^{d,e,f,g}$	$56.47 \pm 1.18^{c,d,e,f,g}$	$69.41 \pm 3.11^{d,e,f,g,h}$	4.22
10	$34.12 \pm 3.11^{c,d,e}$	$36.86 \pm 1.80^{e,f,g,h}$	$56.37 \pm 3.90^{c,d}$	$59.81 \pm 2.85^{c,d,e}$	$73.22 \pm 2.50^{c,d,e,f}$	3.99
11	$18.63 \pm 1.70^{h,i,j,k}$	$32.84 \pm 0.85^{g,h,i}$	$41.67 \pm 3.40^{h,i}$	$44.29 \pm 0.79^{i,k}$	$53.92 \pm 0.85^{k,l}$	5.28
12	$24.51 \pm 2.25^{f,g,h,i}$	$28.80 \pm 2.21^{i,j}$	$49.02 \pm 3.70^{d,e,f,g,h}$	$51.47 \pm 1.47^{f,g,h,i,j}$	$61.76 \pm 2.55^{h,i,j,k}$	4.70
13	$35.52 \pm 3.28^{c,d}$	$35.74 \pm 2.17^{f,g,h}$	$43.14 \pm 3.40^{g,h,i}$	$53.92 \pm 0.85^{e,f,g,h}$	$63.94 \pm 3.62^{g,h,i,j}$	4.53
14	$25.78 \pm 2.78^{f,g}$	$31.22 \pm 2.98^{h,i}$	$46.11 \pm 2.22^{f,g,h}$	$52.89 \pm 0.77^{e,f,g,h,i}$	$70.10 \pm 2.57^{d,e,f,g,h}$	4.47
15	$21.78 \pm 0.77^{g,h,i,j,k}$	$36.34 \pm 2.93^{e,f,g,h}$	$40.41 \pm 2.66^{f,g,h}$	$44.44 \pm 3.56^{j,k}$	44.88 ± 3.36^{m}	5.73
16	$29.78 \pm 2.04^{d,e,f}$	$37.84 \pm 1.35^{d,e,f,g}$	$45.33 \pm 3.53^{f,g,h}$	$50.45 \pm 2.07^{g,h,i,j}$	$54.22 \pm 0.77^{k,l}$	4.91
17	$25.71 \pm 2.47^{f,g}$	$29.28 \pm 2.81^{i,j}$	$34.76 \pm 0.82^{i,j}$	41.43 ± 1.43^{k}	$52.38 \pm 2.97^{l,m}$	5.66
18	$18.10 \pm 1.65^{i,j,k}$	$28.10 \pm 0.82^{i,j}$	32.86 ± 1.43^{j}	$45.32 \pm 3.6^{i,j,k}$	$56.19 \pm 1.65^{j,k,l}$	5.39
19	$23.33 \pm 0.83^{f,g,h,i,j}$	$36.94 \pm 0.78^{e,f,g,h}$	$40.95 \pm 4.36^{h,i,j}$	$53.81 \pm 1.65^{e,f,g,h}$	$61.90 \pm 0.82^{h,i,j,k}$	4.69
20	$28.38 \pm 2.34^{e,f,g}$	$36.04 \pm 2.06^{e,f,g,h}$	$52.70 \pm 1.35^{d,e,f}$	$56.62 \pm 4.40^{c,d,e,f,g}$	$67.12 \pm 2.74^{e,f,g,h}$	4.29
BHA	72.73 ± 2.25^{a}	78.79 ± 0.75^{a}	79.74 ± 2.8^{a}	84.43 ± 2.17^{a}	85.80 ± 3.57^{a}	2.22
BHT	57.97 ± 1.45^{b}	63.29 ± 0.84^{b}	$69.08 \pm 3.01^{b,c}$	85.02 ± 0.83^{a}	$84.05 \pm 2.90^{a,b}$	2.92

* Values (mean value ± standard deviation, n:3) having the same letter(s) in the same column are not significantly different according to Tukey's multiple range test at $p < 0.05$,; SC_{50}: The half-maximal scavenging concentration.

and root induction studies mentioned above. To ensure the ideal initial humidity (85–90 %) in the climate room for plantlets, the plastic pots were covered with breathable polythene bags during the first week. From the second week, the polyethylene bags were gradually removed from the plastic pots in order to reduce the relative humidity to 70 %. The 87 % survival rate achieved in this application after 4 wk was clear evidence that the first acclimatization process was carried out correctly (Fig. 1e). The healthy plantlets that successfully completed the first acclimatization step were relocated to greenhouse conditions first and later to botanical garden conditions after 4 wk, respectively (Fig. 1f and g). The survival percentages of *T. baytopianum* plantlets were 85 % for greenhouse conditions and 75 % for botanical garden conditions. No visible morphological variations were observed when the *in vitro* produced plantlets were compared with naturally growing plants.

3.6. Antioxidant activity

The antioxidant activity of the ethanolic extracts of the samples (1-20) was evaluated at five specified concentrations: 100, 150, 200, 250, and 300 μg/ml, using the DPPH radical scavenging activity method. The results revealed that samples 3 and 4 (76.13 % ± 0.71, 79.84 % ± 3.11 at 300 μg/ml, respectively) exhibited statistically significant similar activity to the standard antioxidant chemicals BHA and BHT (85.80 ± 3.57 and 84.05 ± 2.9 at 300 μg/ml, respectively), ($p > 0.05$). Moreover, these samples demonstrated statistically similar activity with the natural sample of 1 at 250 μg/ml ($p > 0.05$) and the control sample of 2 at all concentrations ($p > 0.05$) except for 250 μg/ml ($p < 0.05$). Compared to the standards of BHA and BHT, the other samples displayed a range of antioxidant activity from moderate to high. Notably, among the samples, sample 15 exhibited the least antioxidant activity at 44.88 % ± 3.36 at 300 μg/ml (Table 4).

Additionally, the extract yields of the samples were calculated, and the results were found to be in the range of 5.10–7.89 %. Samples 1 and 7 showed the least yield, while sample 18 showed the highest yield among the samples. We did not find a significant relationship between the propagation conditions and the extract yields of the samples ($p < 0.05$).

3.7. Antimicrobial activity

According to Kruskal-Wallis analysis, inhibition zone diameters differ between groups. Accordingly, pairwise comparison analysis was chosen from non-parametric tests to understand which two groups the difference was between, and as a result, it was seen that all groups showed lower antimicrobial activity than gentamicin antibiotic ($p < 0.05$). Since the sig value is less than 0.05, there is a statistical difference between the groups. Here, the pairwise comparison test Mann-Whitney U was used to understand which group was superior. Since the sig value is less than 0.05, there is a statistical difference between the groups. Here, the pairwise comparison test Mann-Whitney U was used to understand which group was superior. As a result of the Mann-Whitney U test for pairwise comparison, the test was significant, and the difference was determined in favor of which group by looking at the mean rank values. However, when the test samples were evaluated individually, samples 4 and 10 showed the lowest level of antimicrobial activity, while sample 20 and 1 had the highest antimicrobial activity X^2=58.528. It is seen that sample 4 differs from sample 20 and 1 at a significance level of $p < 0.05$, and sample 10 differs from sample 20 and 1 at a significance level of $p < 0.05$. The results are given in Table 5.

Although sample 20 showed the highest total antimicrobial activity statistically, the antimicrobial activity of sample 1 is remarkable. Except for sample 20, no sample showed antimicrobial activity against *B. cereus*, and in this sample, a high antimicrobial activity (22 mm) was observed. On the other hand, all other samples showed medium and high levels of antimicrobial activity against *P. aeruginosa*, while in sample 20, no antimicrobial activity was observed against *P. aeruginosa*.

4. Discussion

Most species of the family Asteraceae are considered ornamental plants due to their unique flower structures, as well as having medicinal value due to their phytochemical components (Ćavar Zeljković et al., 2015; Piątkowska et al., 2022). These positive features make these plants an obvious target for direct or indirect use by human consumption. Plant losses or extinctions depending on these uses, that is, anthropogenic effects such as a controlled over-harvesting from natural populations, trampling, fragmentation, overgrazing,

Table 5
The antimicrobial activities of the samples of *T. baytopianum* based on Kirby-Bauer disc diffusion (mean value ± standard deviation).

Samples	*B. cereus* (ATCC® 10876)	*S. aureus* subsp. *aureus* (ATCC 6538)	*M. luteus* (NRRL B-1018)	*P. aeruginosa* (ATCC® 27853)	*S. enterica* subsp. *enterica* (ATCC14028)	*K. pneumoniae* subsp. *pneumoniae* (ATCC® 13883)	*S. cerevisiae* (ATCC® MYA-796™™)
1 (*in vivo* plants from natural population)	-	15.63 ± 0.03	22.66 ± 0.13	15.57 ± 0.09	18.64 ± 0.08	17.81 ± 0.03	15.48 ± 0.13
2 (*in vitro* plants without PGRs)	-	17.33 ± 0.03	15.16 ± 0.02	18.02 ± 0.01	11.53 ± 0.16	19.26 ± 0.07	14.00 ± 0.03
3	-	14.00 ± 0.02	8.21 ± 0.03	20.29 ± 0.07	-	18.05 ± 0.01	10.46 ± 0.04
4	-	11.07 ± 0.03	11.66 ± 0.18	18.18 ± 0.23	-	12.65 ± 0.08	10.44 ± 0.06
5	-	18.30 ± 0.11	15.45 ± 0.10	17.24 ± 0.04	-	13.94 ± 0.02	22.38 ± 0.14
6	-	11.53 ± 0.15	17.18 ± 0.15	22.13 ± 0.01	-	7.11 ± 0.04	17.95 ± 0.07
7	-	15.07 ± 0.09	14.86 ± 0.14	17.82 ± 0.10	-	11.24 ± 0.32	14.14 ± 0.45
8	-	14.49 ± 0.15	14.95 ± 0.08	14.46 ± 0.05	15.35 ± 0.01	17.13 ± 0.04	11.67 ± 0.01
9	-	21.68 ± 0.16	13.12 ± 0.07	18.65 ± 0.04	-	17.99 ± 0.35	13.46 ± 0.22
10	-	18.40 ± 0.15	-	14.60 ± 0.02	-	14.12 ± 0.09	17.15 ± 0.02
11	-	14.74 ± 0.41	15.16 ± 0.05	16.75 ± 0.01	-	11.58 ± 0.07	15.56 ± 0.18
12	-	13.76 ± 0.12	10.73 ± 0.13	18.03 ± 0.32	-	8.23 ± 0.01	21.80 ± 0.02
13	-	17.84 ± 0.18	15.10 ± 0.02	20.52 ± 0.02	-	14.02 ± 0.12	20.53 ± 0.13
14	-	20.64 ± 0.12	16.73 ± 0.31	17.36 ± 0.14	-	13.10 ± 0.09	17.92 ± 0.28
15	-	18.57 ± 0.17	14.73 ± 0.21	20.31 ± 0.08	-	9.11 ± 0.04	19.0 ± 0.17
16	-	21.74 ± 0.11	15.30 ± 0.01	16.99 ± 0.08	-	9.84 ± 0.06	22.54 ± 0.08
17	-	18.17 ± 0.13	17.70 ± 0.07	17.38 ± 0.02	-	7.93 ± 0.02	23.65 ± 0.09
18	-	17.45 ± 0.26	15.76 ± 0.09	19.78 ± 0.08	-	9.16 ± 0.03	18.89 ± 0.06
19	-	16.24 ± 0.10	16.10 ± 0.05	14.26 ± 0.2	-	-	17.82 ± 0.05
20	22 ± 0.05	22 ± 0.05	48.00 ± 4.63	-	12 ± 0.12	15 ± 0.05	14 ± 0.03
70 % ethanol	-	-	-	-	-	-	-
Gentamycin	37.58 ± 0.16	40.55 ± 0.11	49.02 ± 0.05	35.16 ± 0.06	44.32 ± 0.02	21.95 ± 0.07	NT
NYS100	NT	NT	NT	NT	NT	NT	17.78 ± 0.08

no antimicrobial activity, (–) Not tested, zone diameter (mm) 6–10 average inhibition, 11–18 good inhibition, > 18 very good inhibition, *Bacillus cereus* ATCC® 10876, *Staphylococcus aureus* subsp. *aureus* ATCC 6538, *Micrococcus luteus* NRRL B-1018, *Pseudomonas aeruginosa* ATCC® 27853, *Salmonella enterica* subsp. *enterica* ATCC14028, *Klebsiella pneumoniae* subsp. *pneumoniae* ATCC® 13883, *Saccharomyces cerevisiae* ATCC® MYA-796™™.

urbanization, dam and road construction are now minimized with modern biotechnological approaches (Cuce et al., 2022; Inceer et al., 2022). In addition, extinction is very rapid, especially in endemic plant species of this family, due to the medicinal phytochemicals they contain. The advantages offered by plant tissue cultures are a solution to this rapid extinction and allow the *ex situ* conservation and production of plantlets with similar genetic characteristics and more effective biological potential as plants growing in natural populations (Cuce et al., 2019; Cuce and Karaismailoğlu, 2023; Nazir et al., 2021). Although there are some studies about the establishment of *in vitro* production protocols in other Asteraceae family members (Catalano et al., 2022; Dimitrova et al., 2018), far fewer of them have revealed the genetic fidelity and biological potential of *in vitro* produced plantlets comparing the naturally growing plants (Chakraborty et al., 2023; Mantovska et al., 2023). In addition, the available literature data revealed that we do not have enough information on the *in vitro* production protocol of *T. baytopianum* and the comparison of the genetic stability, antioxidant and antimicrobial effects of the obtained *in vitro* derived shoots and naturally growing plants.

The sterilization method applied to the initiation material (achene) of *T. baytopianum* was 100% successful and the achenes reached 76.7% germination performance in PGRs-free MS medium. This was a remarkable result for achenes of the family Asteraceae, which have low germination ability in natural populations (Leyva-Peralta et al., 2019). In sterilization processes carried out on different plant species, varied results were obtained from ours depending on the explant type, sterilization agent, concentration, duration, light regime, basal medium and tested PGRs (Cuce at al., 2017; Debnath and Kumaria, 2023). In addition, 6-BA, KIN and 2iP were tested in establishing *in vitro* production protocols of other species of Asteraceae (Cuce et al., 2022; Cuce and Inceer 2024; Inceer et al., 2022; Yordanova et al., 2017). Compare the advantages of 6-BA, such as high activity, more stability and the capacity to metabolize easily in plant tissues to these studies from our results were parallel (Agarwal et al., 2015). In *T. baytopianum*, 6-BA combined with IBA or NAA reached maximum performances in terms of the mean shoot number, node number, callus formation, fresh and dry weight values per explant. Similarly, the strong performance of 6-BA towards the production of multiple shoots, node number and biomass increase (callus formation, fresh and dry weight) for *Erodium hoeftianum* (Cuce, 2021), multiple shoots and node number for *Thlaspi cariense* (Cuce and Karaismailoglu, 2023) and multiple shoots for *Tripleurospermum fissurale* (Cuce et al., 2022), and *Tripleurospermum ziganaense* (Cuce and Inceer, 2024) were reported. The increase in shoot lengths was higher in MS media supplemented with 2iP as opposed to 6-BA. Although the highest effect of mean shoot length per explant (74.7 mm) was obtained from MS media enriched with 0.5 mg L^{-1} 2iP plus 0.1 mg L^{-1} NAA, there was also a significant increase in 2iP media combined with IBA. In line with our results, the researchers tested the effects of the three cytokinins tested in our study on shoot length in many different plant species and reported that 2iP was more effective (Cuce et al., 2017; Ranganatha et al., 2020; Song et al., 2021). It has also been reported among members of the same genus that the effect of cytokinin on the shoot length parameter varies depending on the studied species (Cuce et al., 2022; Cuce and Inceer, 2024; Inceer et al., 2022).

Auxins tested in this study achieved 100 % rooting success at different concentrations. In terms of average number of roots per shoot, IBA (0.5 mg L^{-1}) showed the highest effect with 10.8. Apart from this, although the control application exhibited a lower rooting percentage than all concentrations of the two auxin types tested, the average root length (100.8 mm per shoot) and number of secondary roots (10.9 per shoot) were more ideal in their formation. As in our study, IBA, NAA or IAA have been tested in many plant species for strong root formation of shoots under *in vitro* culture conditions (Chakraborty et al., 2023; Inceer et al., 2022; Song et al., 2021). Strong effect of IBA towards induction of roots was reported for *Jasminanthes tuyetanhiae* (Nam et al., 2022) and *T. fissurale* (Cuce et al., 2022). Studies have reported that IBA either has no effect (Cuce, 2019) or is less effective than NAA (Chakraborty et al., 2023), depending on the studied species, auxin concentration, and basal medium. Control

treatments alone were sufficient for the rooting of *T. cariense* species *in vitro* MS media (Cuce and Karaismailoglu, 2023). These findings presented similarities and differences between the results of our current study and those of aforementioned above studies in terms of the tested auxin for rooting.

Determining the genetic stability of micropropagated plants is important for the production of uniform plants with cytogenetically similar ancestral character. *Tripleurospermum baytopianum* is a diploid species with $2n = 18$ chromosomes (Inceer et al., 2018). The present results obtained from cytogenetic analysis indicated that all of the plants propagated *in vitro* were diploid with $2n = 18$ chromosomes as in the mother plants (Fig. 1d). It can be inferred that there was no change in chromosome number of the propagated plants, and hence the genetic stability remained preserved in the propagated plants, wherein confirmation of the genetic stability is a critical prerequisite for any genotype specific mass multiplication protocol.

The DPPH scavenging activity method measures a substance's ability to neutralize the DPPH free radical, offering an accurate way to determine its antioxidant potentials. Antioxidants are substances that can give hydrogen atoms or electrons that neutralize free radicals. This idea is employed by the DPPH assay to evaluate an antioxidant's capacity to scavenge the DPPH radical (Brand-Williams et al., 1995). DPPH scavenging activity (%) of the samples of *T. baytopianum* (1-20) and standard antioxidants of BHA and BHT is displayed in Table 4. A statistical evaluation was performed for each concentration and as a result, statistically significant similarities or differences were found between some samples. According to the present results, the statistical evaluation indicates that the concentrations of plant growth regulators and the concentrations of the samples in the antioxidant activity assay, significantly affect the DPPH scavenging activity (%) of the samples ($p < 0.05$). The most active samples are 3 and 4, which shows supplement of 6-BA and IBA together, at concentrations of 0.25, 0.1 mg L^{-1}, and 0.5, 1 mg L^{-1}, respectively for *in vitro* growth of *T. baytopianum* has enhancing effect on antioxidant activity. The least active extract is sample 15, micropropagated with KIN and IBA at concentrations of 0.25 and 0.1 mg L^{-1}. SC_{50} indicates the amount of antioxidant required to produce a 50% inhibition of free radicals. The SC_{50} values for samples 3 and 4 are 3.51 and 3.73 μg/ml, respectively. These values are comparable to those of BHA and BHT, which are 2.22 and 2.92 μg/ml. Furthermore, when comparing the antioxidant activity of the sample 2 (control) with samples 3 and 4, *in vitro* derived shoots of *T. baytopianum* using PGRs does not enhance the antioxidant capacity of the samples except for 250 μg/ml ($p > 0.05$). However, natural sample (negative control) of 1 showed less activity than the positive control sample of 2 at 100 and 150 μg/ml ($p < 0.05$) and similar activity with the control sample of 2 at 200, 250 and 300 μg/ml concentrations ($p > 0.05$).

A thorough antioxidant activity study contrasting natural and micropropagated plantlets of *T. baytopianum* has not been carried out, despite the fact antioxidant activity of most members of *Tripleurospermum* from natural populations without *in vitro* cultivation. Colak et al. (2017) studied the antioxidant activities of many taxa of *Tripleurospermum* from natural populations in Türkiye and showed their high antioxidant capacity that ranges from 45.26 ± 0.05 to 135.90 ± 12.67 μmol Trolox equivalents per 100 g dry weight in the studied taxa. Likewise, Chen et al. (2022) researched antioxidant capacity of *Tripleurospermum limosum* ethanol extract and found the SC_{50} value as 33.30 ± 0.2 μg/ml in this species. In this study, we showed that ethanol extract of *T. baytopianum* has more antioxidant capacity than *T. limosum* with the SC_{50} value of 3.51 μg/ml (sample 3).

The antioxidant activities both of natural and micropropagated plantlets were also conducted on some members of Asteraceae (Minutolo et al., 2012; Rasool et al., 2012; Rocchi et al., 2024). A recent research showed that compared to micropropagated infusions, the wild infusions of *Artemisia eriantha* exhibited greater levels of phenolic compounds and antioxidant activity before digestion (Rocchi et al., 2024). In another research, two Aster species -*Aster sedifolius* and *Aster caucasicus*- callus cultures were established because of their high antioxidant component content. The induction and growth rate of callus from leaf, petiole, and root explants are examined in relation to growth media and light conditions. The results showed that antioxidant chemicals were created by Aster callus cultures (Minutolo et al., 2012). Also, a study was carried out to evaluate the callus derived from *Artemisia amygdalina* Decne nodal segments and leaf explants' capacity to scavenge free radicals. The explants received inoculation on MS media supplemented with different BAP, KIN, NAA, and 2,4-D (2,4-dichlorophenoxyacetic acid) concentrations in order to induce callus. In the DPPH and deoxyribose experiment, methanol and aqueous extracts exhibited greater scavenging activity (Rasool et al., 2012). In nature, plants frequently have to deal with stresses including pests, illnesses, and environmental factors. Antioxidant production might increase as a protective response to this stress. However, in micropropagation, it is occasionally achievable to optimize the use of particular growth media and PGRs therapies to increase antioxidant levels. In light of all these studies and our results in this study, it can be concluded that the antioxidant capacities of these plants may vary depending on the conditions of the growing environment.

This study shows that the contents and amounts of cytokinin and auxin used in the cultivation of *T. baytopianum* could have an effect on secondary metabolite production. Phenolic compounds begin to be produced in plants due to environmental stress factors (Zagoskina et al., 2023). These reasons may account for the relatively high antimicrobial activity in plants collected from natural environments. On the other hand, PGRs are molecules naturally produced by plants in the growth and differentiation of plants that have effects in alleviating different abiotic and biotic stresses (Akhtar et al., 2020). It is also known that relatively high amounts of cytokinin in plants increase resistance to pathogens (Akhtar et al., 2020). This may be the reason for the high antimicrobial activity against pathogens in sample 20 (1.0 mg L^{-1} 2iP). Similarly, the moderate antimicrobial activity of sample 8 (1.0 mg L^{-1} KIN) against *S. enterica* subsp. *enterica* may be due to the high amount of cytokinin.

The limited studies are showing that the antimicrobial activity of plants grown in nature is higher than that of cultivated plants (Cuce et al., 2017; 2019; Cuce and Basançelebi, 2021). The *Tripleurospermum* species, numerous chemical compounds have been identified and classified into distinct classes, predominantly encompassing terpenes, hydrocarbons, steroids, hydrocarbons, oxygenated compounds, flavonoids, tannins, alcohols, organic acids, melatonin, and fragrant compounds (Marakhova et al., 2024; Sheydaei and Duarte, 2023). Tannins extracts can inhibit the growth of *Esherichia coli, P. aeruginosa, S. aureus, Aspergillus niger* and *Candida albicans* (Mailoa et al., 2014). Flavonoids possess antifungal, antiviral, and antibacterial activity (Cushnie and Lamb, 2005).

The findings from the report by Šibul et al. (2020) highlight the presence of bioactive compounds within the *Tripleurospermum inodorum* that possess significant medicinal properties. On the other hand, there are a few studies on the antimicrobial activity of some medicinally important Asteraceae plants, but a comprehensive study comparing natural and micropropagated *T. baytopianum* plantlets has not been carried out until now. Erdoğan et al. (2013) reported that *T. parviflorum* showed the highest antimicrobial activity on *S. aureus* and also had antibacterial activity on *P. aeruginosa*, albeit at a lower level. Tofighi et al. (2015) reported in their study that the extract of *T. disciforme*, another species of the genus *Tripleurospermum*, showed antimicrobial activity on *S. aureus* and *S. epidermidis*, but was not effective on *B. cereus* and *P. aeruginosa*. The results of this study are broadly consistent with our study. Marakhova et al. (2024) observed that ethanol extract of *T. inodorum* had remarkable effects on *S. aureus* (22 mm). That study also revealed that lower flavonoids

in extracts obtained from plants collected during butonisation and flowering. On the other hand, in the majority of studies on the antimicrobial activity of plants obtained by *in vitro* and *ex vitro* methods, antimicrobial activity against *S. aureus* bacteria has been reported. When the literature is examined, it is seen that Gram-positive bacteria are more sensitive to antibacterial agents in antimicrobial activity studies (Bendjeddou et al., 2016; Çil et al., 2020).

5. Conclusion

In this study, an efficient and rapid micropropagation protocol has been developed for threatened endemic species *T. baytopianum* for the first time. Besides, the obtained data from the cytogenetic and biochemical studies revealed that the genetic and metabolic stabilities of the propagated plants. On the other hand, it could be seen that antimicrobial activity is affected by PGRs concentrations, and thus it can be said that the abundance of stress factors in nature positively affects the production of secondary metabolites such as antimicrobial agents in plants. The data presented herein will also be useful for conservation plans of other threatened endemic species of *Tripleurospermum* as well as other different threatened endemic species.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Mustafa Cuce: Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation. **Huseyin Inceer:** Writing – review & editing, Writing – original draft, Software, Methodology, Data curation. **Emine Bagdatli:** Writing – review & editing, Writing – original draft, Software. **Ömer Ertürk:** Writing – review & editing, Writing – original draft. **Elif Çil:** Writing – review & editing, Writing – original draft. **Rabia Sagan:** Methodology.

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