doi:10.14720/aas.2025.121.3.18920 Original research article / izvirni znanstveni članek

Successful micropropagation of critically endangered *Thymus bovei* Benth: A wild medicinal plant from the Jordanian environment

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Received June 10, 2024, accepted September 02, 2025
Delo je prispelo 10. junij 2024, sprejeto 2. september 2025

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Abstract: *Thymus bovei* Benth. (Lamiaceae), locally referred to as Zateer Barry, is a critically endangered native plant species in Jordan (Taifour and EL-Oqlah, 2014), renowned for its exceptional medicinal properties. This study aims to establish a reliable micropropagation technique to prevent *T. bovei* from extinction. The research focused on explant establishment, shoot multiplication, rooting, callus induction, and acclimatization of *T. bovei*. Explants were established in a half-strength Murashige and Skoog (MS) media supplemented with different concentrations of gibberellic acid (GA_3). Successful shoot multiplication was achieved by subculturing nodal segments onto half-strength MS medium supplemented with kinetin (KIN), thidiazuron (TDZ), or benzylaminopurine (BA). Among the treatments, the most effective medium for shoot multiplication was supplemented with 2.5 mg l^{-1} KIN and 0.5 mg l^{-1} GA_3, which resulted in an average of 7.50 ± 0.54 microshoots per explant and a shoot length of 2.33 ± 0.18 cm. Rooting was most effective on half-strength MS media with 1.0 mg l^{-1} indole-3-butyric acid (IBA), yielding an average of 5.29 roots per microshoot. The highest callus development, measured by fresh mass (0.601 g), occurred in half-strength MS media with 2.0 mg l^{-1} 2,4-D, while no callus formed with naphthaleneacetic acid (NAA). Acclimatized plants showed a 90 % survival rate when transferred to greenhouse conditions.

Key words: endangered plant; Jordan; medicinal plant; micropropagation; tissue culture; *Thymus bovei*

Uspešna razmnožitev kritično ogrožene vrste materine dušice (*Thymus bovei* Benth), samonikle zdravilne rastline iz Jordanije

Izvleček: Vrsta materine dušice, *Thymus bovei* Benth., (Lamiaceae), lokalno imenovana kot 'Zateer Barry' je kritično ogrožena samonikla zdravilna rastlina v Jordaniji, poznana zaradi izrednih zdravilnih lastnosti. V raziskavi je predstavljena zanesljiva tehnika njene mikropropagacije, da bi preprečili njeno izumrtje. Poudarek raziskave je bil na ohranjanju izsečkov, indukciji kalusa, nastajanju, vkoreninjanju in aklimatizaciji poganjkov. Izsečki so bili gojeni na polovičnem Murashige in Skoogovem (MS) gojišču, ki so mu bile dodane različne koncentracije giberilinske kisline (GA_3). Uspešen razvoj poganjkov je bil dosežen pri gojenju nodijskih segmentov na polovičnem MS gojišču, ki so mu dodali kinetin (KIN), tidiazuron (TDZ) ali benzilaminopurin (BA). Med obravnavanji je bila najbolj učinkovita tvorba poganjkov na gojišču, ki so mu dodali 2,5 mg l^{-1} KIN in 0,5 mg l^{-1} GA_3, ko je v poprečju nastalo 7,50 ± 0,54 mikropoganjkov na izseček, z dolžino poganjka 2,33 ± 0,18 cm. Vkoreninjanje je bilo najbolj učinkovito na polovičnem MS gojišču z dodatkom 1,0 mg l^{-1} indol-3-maslene kisline (IBA), kar je v poprečju dalo 5,29 korenin na mikropoganjek. Največja tvorba kalusa, merjena kot sveža masa (0,601 g), je nastala na polovičnem MS gojišču z dodatkom 2,0 mg l^{-1} 2,4-D, medtem, ko se kalus pri dodatku naftalenocetne kisline (NAA) sploh ni tvoril. Aklimatizirane rastline so imele pri prenosu v rastlinjak 90 % preživetje.

Ključne besede: ogrožene rastline, Jordanija, zdravilne rastline; mikropropagacija, tkivna kultura, *Thymus bovei*

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1 INTRODUCTION

In recent years, a surge in global interest in traditional medical systems has been noticed. According to current projections, developing countries account for the vast majority of users worldwide in terms of their dependence on medicinal plants to meet health needs (Al-Qura'n, 2011). Despite the availability of modern medicine in developing countries, the use of medicinal herbs (phytomedicines) persists due to popular and historical culture (Karim and Al-Qura'n, 1986). Jordan is distinguished by its rich plant diversity, owing to its small size; it contains nearly 2530 wild plant species, (Al-Qura'n, 2011; Royal Botanic Garden (RBG), 2012). The Jordan flora has 485 medicinal plant species divided into 330 genera and 99 families (Al-Eisawi, 1996).

Thymus bovei is known as (Zateer Barry) in the Middle East. It is a medicinal and fragrant perennial herb of the genus *Thymus* with a variety of medicinal and culinary uses. Due to its remarkable medicinal properties, *T. bovei* is known as one of the most famous thyme species (Hassan et al., 2018). This has been attributed to the anthelmintic, expectorant, antispasmodic, and antiseptic properties of its essential oil, which was prescribed since ancient times to cure respiratory, skin, and blood infections (Tepe et al., 2011; Abdel-Hady et al., 2014). *T. bovei* essential oil contains phytochemical compounds such as thymol, carvacrol and trans-geraniol and has good antioxidant, antibacterial, and anthelmintic properties (Jaradat et al., 2016). *T. bovei* is found in different regions of Jordan including Irbid, Petra, Mafraq, Azraq, and Tafila and it has been observed in some localities but not in any protected area in Jordan, where this species is subject to excessive collection by people for food and medicinal uses (Taifour and EL-Oqlah, 2014).

One of the most important modern techniques for preserving plants and their genetic resources is tissue culture (Engelmann and Engel, 2002; Shibli, et al., 2006). Now micropropagation is the alternative effective method of plant propagation leaving behind the conventional propagation methods. *In vitro* propagation will be suitable for fast plant proliferation, removal of pathogens, germplasm preservation, and production of secondary metabolites (Negash, et al., 2001; Ahmad, 2013); Shatnawi, et al., 2006). Conservation via tissue culture provides a powerful and diverse set of procedures that can be used when other conservation strategies are not possible. *In vitro* culture of endangered plants offers several advantages, including the capacity to produce species with limited reproductive potential and those found in vulnerable environments (Fay 1992). Previous investigations of *Thymus* sp. using i*n vitro* techniques have been industrialized, including *Thymus spicata* L.*, T. vulgaris* L.*, T. longicaulis* C.Presl and *T. lotocephalus* G.López & R.Morales (Ozudogru et al. 2011; Coelho et al., 2012; Tahtamouni et al., 2016).

Plant growth regulators (PGRs) play a pivotal role in regulating developmental processes during micropropagation (Sabagh et al., 2021). Among these, auxins, cytokinins, and gibberellins are the most commonly used. Auxins such as indole-3-butyric acid (IBA), naphthalene acetic acid (NAA), and indole-3-acetic acid (IAA) are essential for root initiation and callus formation (Elmongy et al., 2018). Cytokinins, including benzylaminopurine (BA), kinetin (KIN), and thidiazuron (TDZ), stimulate cell division and promote shoot induction and multiplication (Ali et al., 2022). Gibberellins, particularly gibberellic acid (GA_3), are mainly involved in shoot elongation and enhancing overall plantlet vigor (Shah et al., 2023). The interaction and concentration of these hormones greatly influence the success of each micropropagation stage, including explant establishment, shoot multiplication, rooting, and acclimatization (Grzelak et al., 2024).

Due to the significance of *T. bovei* as a medicinal herb, and because it is one of the many rare plants found in only a few locations within the Jordanian environment, this study was conducted to develop a reliable protocol for its preservation. *T. bovei* is native to the eastern Mediterranean region and, unfortunately, is subject to severe overharvesting and the impacts of climate change in Jordan. The aim of this study was to establish a dependable method for the mass propagation of *T. bovei* through tissue culture, one of the most widely adopted in vitro conservation techniques for maintaining plant genetic resources.

2 MATERIALS AND METHODS

Research was carried out at the Hamdi Mango Center for Scientific Research (HMCSR) in the plant tissue culture labs of the University of Jordan. The plant samples (aerial parts) were gathered from a naturally growing solitary plant found in Almafraq, Jordan, (Latitude: 315282E, Longitude: 355425N) (Figure 1).

2.1 ESTABLISHMENT OF *THYMUS BOVEI* EXPLANTS

Nodal segments of *Thymus bovei* were subjected to surface sterilization to ensure aseptic conditions for in vitro culture. Initially, the explants were washed with a few drops of mild detergent and rinsed thoroughly under running tap water for 15 minutes to remove surface debris. The segments were then immersed in a 1.5 % (v/v)

Figure 1: Wild *Thymus bovei* collected in Almafraq, Jorden, (Latitude: 315282E, Longitude: 355425N). Blue bar represents 10 cm.

sodium hypochlorite (NaOCl) solution for 10 minutes under a laminar airflow cabinet. Afterward, they were rinsed three times with sterile distilled water, with each rinse lasting 5 minutes. This was followed by a 30 second immersion in 70 % ethanol (v/v), and finally, the explants were again rinsed three times with sterile distilled water (5 minutes each) under aseptic conditions in the laminar airflow cabinet. The sterilized explants were transferred into Erlenmeyer flasks containing 100 ml of half-strength solid Murashige and Skoog (MS) medium (Murashige & Skoog, 1962), supplemented with 30 g l^{-1} sucrose and 8 g l^{-1} agar. Different concentrations of gibberellic acid (GA_3) (0, 0.5, and 1.0 mg l^{-1}) were added to the medium to promote bud growth and to determine the optimal GA_3 concentration for shoot multiplication when combined with other micropropagation growth regulators. The rate of shoot emergence was measured after 4 weeks. Cultures were grown under specific conditions (16/ 8 h of light/dark period at 24 ±2 °C).

2.2 SHOOT MULTIPLICATION OF *THYMUS BOVEI*

Two-centimeter nodal segments (each with two nodes) from previously established tissue culture plants were placed in 100 ml of MS medium (Murashige and Skoog, 1962), supplemented with 30 g l^{-1} sucrose, 8 g l^{-1} agar, and varying concentrations (0.0, 0.5, 1.0, 1.5, 2.0, 2.5, or 3.0 mg l^{-1}) of 6-benzyladenine (BA), kinetin (KIN), or thidiazuron (TDZ), along with 0.5 mg l^{-1} GA_3. The explants were cultured for four weeks at 24 ± 2 °C under a 16/8 h (light/dark) photoperiod. Growth parameters, including the number of microshoots and shoot height, were recorded after four weeks of culture.

2.3 ROOTING OF *THYMUS BOVEI*

Shoot tips were subcultured in solid MS media containing varying concentrations (list here from scheme) of auxin indole acetic acid (IAA), or indole-3-butyric acid (IBA), or 1-naphthalene-acetic acid (NAA). The cultures were kept at 24 ± 2 °C for four weeks, under 16 hours of light and 8 hours of darkness. Growth parameters, including root number, root length, and shoot height, were recorded after four weeks of incubation with the corresponding growth regulators.

2.4 CALLUS INDUCTION AND MULTIPLICATION OF *THYMUS BOVEI*

Shoot tips were transferred to solid MS media supplemented with 3 % sucrose, and 0.8 % agar, with different concentrations of auxins 1-naphthalene-acetic acid (NAA) or 2,4-dichlorophenoxyacetic acid (2,4-D) (0.0, 1.0, 2.0, and 3.0 mg l^{-1}). Half of the cultures were grown under 16-hour light/8-hour dark cycle, while the other half were grown under dark conditions. The cultures were maintained at 24 ± 2 ^{0}C for 6 weeks. After six weeks, the callus was harvested for determination of fresh weight.

2.5 EX VITRO ACCLIMATIZATION OF *THYMUS BOVEI*

Once the plantlets had developed roots, they were exposed for 3 days in test tubes in laboratory before transferring to the greenhouse environment where temperatures were maintained at 24 ± 2 during the day and 20 ± 2 °C at night, with intermittent misting. The plantlets were grown in trays filled with 1 part peat moss and 1 part perlite.

2.6 DATA ANALYSIS

In the current study, a completely randomized design (CRD) was applied. For the shooting percentage, one explant was placed per replicate, with a total of 20 replicates for each GA_3 concentration. For shoot multiplication experiments, each treatment was performed 5 times (4 explants per replicate). Rooting experiments

included 10 replicates (one explant per replicate in test tubes), while callus experiments had 10 replicates (2 explants per replicate in petri dishes). For acclimatization experiments, 10 plants were used for each hormone treatment. SPSS software was used to analyze the results. The standard error of the mean was calculated, and analysis of variance (ANOVA) was performed for each experiment. The Tukey HSD test was used to differentiate means at $p \leq 0.05$.

3 RESULT AND DISCUSSION

A comprehensive *in vitro* propagation protocol was successfully developed for *Thymus bovei*, a critically endangered medicinal plant. The study demonstrated that the addition of gibberellic acid (GA_3), particularly at 0.5 mg l^{-1}, significantly improved explant establishment. Among the cytokinins tested, kinetin (KIN) at 2.5 mg l^{-1} yielded the highest shoot multiplication rate and shoot number, outperforming both benzylaminopurine (BA) and thidiazuron (TDZ). For root induction, indole-3-butyric acid (IBA) at 1.0 mg l^{-1} was the most effective, producing the greatest number of roots and root length, while naphthaleneacetic acid (NAA) was ineffective under light conditions. Callus induction was successfully achieved using 2, 4-D at 2.0 mg l^{-1}, whereas NAA failed to induce callus under the same conditions. The regenerated plantlets were successfully acclimatized with a high survival rate, confirming the feasibility of this protocol for conservation and large-scale propagation of *T. bovei*.

3.1 GA_3 ENHANCES SUCCESSFUL IN VITRO ESTABLISHMENT OF *THYMUS BOVEI*

Thymus bovei is a critically endangered plant species that requires efficient propagation methods for its conservation. To improve explant establishment *in vitro*, we tested the effect of different concentrations of gibberellic acid (GA_3) on shoot tip culture. The most important result showed that culturing shoot tips on MS medium supplemented with 0.5 mg l^{-1} GA_3 led to the highest production of new microshoots (90 %) (Table 1, Figure 2). GA_3 is a well-known plant hormone that promotes growth by stimulating seed germination and the transition from meristem to shoot development (Gupta et al., 2013). Similar positive effects of GA_3 on shoot proliferation have been reported in *Thymus satureioides* Coss. (Aicha et al., 2013) and *Mentha* x *piperita* L. (Islam and Alam, 2018). These findings indicate that GA_3 at 0.5 mg/L effectively enhances the *in vitro* establishment of *T. bovei* and supports its micropropagation efforts.

Table 1: Effect of GA3 concentration on the percentage of emerging shoots of *Thymus bovei.*

GA_3 Conc. (mg l^{-1})	Shoots (%)*
0.0	10 ± 1.0 b[x]
0.5	90 ± 10.0 a
1.0	20 ± 1.5 b

*Shooting (%): Represents the mean percentage of explants that produced shoots. One explant was placed per replicate, with a total of 20 replicates for each GA_3 concentration. xThe values presented are means ± standard error, N = 20..

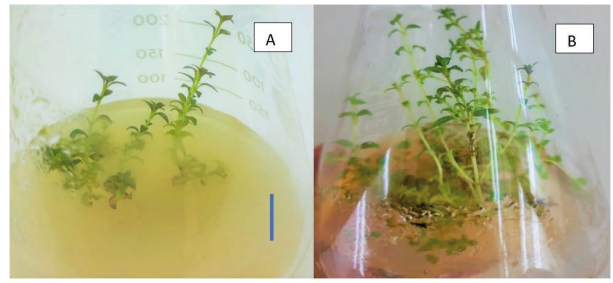

Figure 2: Microshoot formation of *Thymus bovei* cultured on MS medium supplemented with 30 g l^{-1} sucrose and 8 g l^{-1} agar. (A) Control MS medium without growth regulators. (B) MS medium supplemented with 0.5 mg l^{-1} GA_3. Cultures were maintained for 2 weeks at 24 °C under a 16/8 h light/dark photoperiod. The blue bar represents 1.0 cm.

3.2 SHOOT MULTIPLICATION IS SIGNIFICANTLY INFLUENCED BY TYPE AND CONCENTRATION OF CYTOKININS, BA PROMOTES MODERATE SHOOT PROLIFERATION BUT LESS EFFECTIVELY THAN KIN

Cytokinins are widely used to promote shoot multiplication in plant tissue culture. To evaluate the effect of benzylaminopurine (BA) on *Thymus bovei*, nodal segments were cultured on MS medium supplemented with 2.5 mg l^{-1} BA. This treatment resulted in a moderate shoot proliferation rate, producing an average of 4.3 shoots per explant with the shortest shoot length of 2.0 cm (Table 2). Notably, no callus formation was observed under these conditions. BA is known to stimulate protein synthesis, which enhances cell division and growth (Royani et al., 2021). Our findings align with previous reports of BA's role in promoting shoot multiplication in Lamiaceae species, such as patchouli (*Pogostemon cablin* (Blanco) Benth.) (Swamy et al., 2016). Therefore, while BA positively influences shoot proliferation in *T. bovei*, its effect

Table 2: Effect of BA concentration on shoot multiplication in vitro of *Thymus bovei* after four weeks' growth period

BA Conc. (mg $^{-1}$)	Microshoot number/ explant	Shoot length (cm)
0.0	2.85 ± 0.28 c^{x}	3.41 ± 0.10 a
0.5	2.85 ± 0.31 c	2.60 ± 0.07 b
1.0	3.90 ± 0.19 ab	2.40 ± 0.06 bc
1.5	3.85 ± 0.18 ab	2.35 ± 0.08 bcd
2.0	3.80 ± 0.20 ab	2.33 ± 0.18 bc
2.5	4.30 ± 0.21 a	2.00 ± 0.00 d
3.0	3.20 ± 0.26 bc	2.11 ± 0.04 cd

xThe values are means ± standard error, N = 5.

is less pronounced compared to kinetin.

3.3 KIN AT 2.5 MG **L**$^{-1}$ ACHIEVES THE HIGHEST SHOOT MULTIPLICATION RATE

Cytokinins play a crucial role in stimulating shoot proliferation in plant tissue culture. To determine the effectiveness of kinetin (KIN) on *Thymus bovei*, explants were cultured on MS medium supplemented with 2.5 mg l^{-1} KIN. This treatment resulted in the highest shoot multiplication rate, with an average of 7.5 shoots per explant, surpassing the control (Table 3). Additionally, shoot length was significantly increased compared to lower KIN concentrations, and no callus formation was observed. These results are consistent with Teshome et al. (2016), who reported the longest shoot length (6.33 cm) in lemon-scented thyme (*Satureja punctata* (Benth.) R.Br. ex Briq.)) at the same KIN concentration. Similar positive effects of KIN were also documented in kidney tea plants (*Orthosiphon aristatus* (Blume) Miq.))) (Jayakumar et al., 2013). Overall, 2.5 mg l^{-1} KIN is highly effective in promoting shoot multiplication in *T. bovei*.

3.4 TDZ INDUCES SHOOT FORMATION BUT RESULTS IN SHORTER, STUNTED SHOOTS

Thidiazuron (TDZ) is known for its strong cytokinin-like activity in promoting shoot multiplication. To evaluate its effect on *Thymus bovei*, explants were cultured on MS medium supplemented with varying TDZ concentrations. The highest number of microshoots per explant (4.85) was observed at 0.5 mg l^{-1} TDZ (Table 4). However, increasing TDZ concentrations led to a decline in shoot multiplication and significantly reduced shoot length, with the shortest shoots (2.08 cm) recorded at 3.0 mg l^{-1}. While TDZ has shown excellent multiplication performance in some Lamiaceae species, such as wild mint (*Mentha arvensis* L.) where it promoted both high shoot numbers and length (Faisal et al., 2014), it can also have adverse effects. For instance, negative impacts of TDZ were reported in *Sideritis athoa* Papan. & Kokkini.and *Thymus moroderi* Pau ex Martinez

(Papafotiou and Kalantzis, 2009; Marco-Medina and Casas, 2015). These results suggest that although TDZ can induce shoot formation in *T. bovei*, its higher concentrations may lead to stunted growth, limiting its practical application.

3.5 AUXIN TYPE DETERMINES ROOTING EFFICIENCY IN T. BOVEI MICROSHOOTS; NAA SHOWS LIMITED ROOT INDUCTION POTENTIAL IN *T. BOVEI*

Auxins like naphthalene acetic acid (NAA) are often

Table 3: Effect of kinetin level on shoot multiplication in vitro of *Thymus bovei* after four weeks' growth periods

KIN Conc. (mg l^{-})	Microshoot number/ explant	Shoot length (cm)
0.0	2.85 ± 0.28 c^{x}	3.41 ± 0.10 d
0.5	2.85 ± 0.29 c	4.55 ± 0.11 a
1.0	4.30 ± 0.26 b	4.00 ± 0.16 b
1.5	4.60 ± 0.23 b	3.90 ± 0.06 c
2.0	4.70 ± 0.23 b	3.86 ± 0.11 c
2.5	7.50 ± 0.54 a	2.33 ± 0.18 e
3.0	6.50 ± 0.32 b	3.50 ± 0.12 d

x The values presented represent the means ± standard error, N = 5

Table 4: Effect of thidiazuron (TDZ) level on in vitro shoot multiplication of *Thymus bovei* after four weeks' growth period

TDZ Conc. (mg l^{-1})	Microshoot number/explant	Shoot length (cm)
0.0	2.85 ± 0.28 c^{x}	3.41 ± 0.10 a
0.5	4.85 ± 0.58 a	2.08 ± 0.02 c
1.0	4.05 ± 0.32 ab	2.41 ± 0.09 bc
1.5	4.10 ± 0.33 ab	2.48 ± 0.07 b
2.0	3.75 ± 0.41 ab	2.28 ± 0.06 bc
2.5	2.95 ± 0.26 c	2.19 ± 0.04 bc
3.0	2.60 ± 0.31 c	2.32 ± 0.09 bc

x The values presented represent the means ± standard error, N = 5

Table 5: Effect of 1-naphthalene acetic acid (NAA) level on the number of roots, root length, and shoot length, of *in vitro* grown *T bovei* after four weeks.

NAA conc. (mg l^{-1})	Roots number	Roots length (cm)	Shoot height (cm)
0.0	0	0	2.53 ± 0.14 b[x]
0.5	0	0	3.40 ± 0.37 ab
1.0	0	0	3.25 ± 0.24 ab
1.5	0	0	3.69 ± 0.31 a
2.0	0	0	3.41 ± 0.33 ab

[x]The values presented are the means ± standard error, N = 10

Table 6: Effect of indole-3-butyric acid (IBA) level on rooting, root length, and shoot length, of *in vitro* grown *T bovei* after four weeks.

IBA conc. (mg l^{-1})	Roots number	Roots length (cm)	Shoot height (cm)
0.0	0.00 d	0.00 c[x]	2.53 ± 0.14 c
0.5	2.33 ± 0.16 c	0.10 ± 0.0 c	2.98 ± 0.11 bc
1.0	5.29 ± 0.42 a	0.43 ± 0.06 a	6.16 ± 0.64 a
1.5	4.20 ± 0.17 b	0.26 ± 0.02 b	4.26 ± 0.41 b
2.0	2.67 ± 0.06 c	0.27 ± 0.01 b	3.58 ± 0.51 bc

[x]The values presented are the means ± standard error, N = 10

used to induce rooting in plant tissue culture. To assess its effect on *Thymus bovei*, explants were treated with various concentrations of NAA; however, no root formation was observed at any tested concentration (Table 5, Figure 3). These findings are consistent with Teshome et al. (2016), who also reported the absence of rooting in lemon-scented thyme (*Satureja punctata*) under similar NAA treatments. In contrast, Sarropoulou and Maloupa (2019) observed rooting induction at 0.5 mg l^{-1} NAA in *Satureja thymbra* L., indicating that rooting response to NAA may be species-specific within the Lamiaceae family. Interestingly, shoot height increased with higher NAA concentrations, possibly due to enhanced shoot apical dominance, as noted by Teshome and Soromessa (2015) in *Satureja abyssinica* (Hochst. ex Benth.) Briq.. Therefore, while NAA does not promote rooting in *T. bovei*, it may influence shoot elongation.

3.6 IBA AT 1.0 MG L^{-1} INDUCES OPTIMAL ROOTING AND ROOT DEVELOPMENT

Indole-3-butyric acid (IBA) is widely recognized as an effective auxin for promoting root initiation in plant tissue culture. To determine its effect on *Thymus bovei* microshoots, various concentrations of IBA were tested for rooting efficiency. Root formation increased with IBA concentration up to 1.0 mg l^{-1}, where the highest average number

Figure 3: Effect of 2.0 mg l^{-1} NAA on roots of *in vitro* grown *T bovei*. The blue bar represents 2.0 cm.

Figure 4: Effect of 1.0 mg l^{-1} IBA on roots of *T bovei*. The blue bar represents 1.0 cm.

Figure 5: Effect of 1.0 mg l^{-1} IAA on roots of *T bovei*. The blue bar represents 1.0 cm.

of roots per microshoot (5.29) and root length (0.43 cm) were recorded (Table 6, Figure 4). However, concentrations above 1.0 mg l^{-1} caused a significant decline in both root number and length, and the control group without IBA showed no rooting. The positive effect of IBA is attributed to its ability to elevate endogenous auxin levels, enhancing stability and reducing catabolism through conjugation with growth inhibitors (Frick & Strader, 2018). Similar results were reported by Mustafa and Weal (2022) in *Thymus serpyllum* L., where 1.0 mg l^{-1} IBA yielded the highest rooting rates. These findings confirm that 1.0 mg l^{-1} IBA is optimal for inducing rooting and root development in *T. bovei* microshoots.

Table 7. Effect of indole acetic acid (IAA) level on the rooting, root length, and shoot length, of *in vitro* grown *T bovei* after four weeks.

IAA conc. (mg l^{-1})	Roots number	Roots length (cm)	Shoot height (cm)
0.0	0.0 d	0.00 d[x]	2.53 ± 0.14 b
0.5	1.0 ± 0.0 c	0.20 ± 0.0 bc	2.76 ± 0.11 ab
1.0	3.5 ± 0.26 a	0.32 ± 0.04 a	3.18 ± 0.13 a
1.5	3.0 ± 0.0 a	0.30 ± 0.06 ab	2.79 ± 0.26 ab
2.0	2.4 ± 0.17 b	0.15 ± 0.01 c	2.38 ± 0.84 b

[x]The values presented are the means ± standard error, N = 10

3.7 IAA PROMOTES ROOTING BUT LESS EFFECTIVELY COMPARED TO IBA

Indole-3-acetic acid (IAA) is another commonly used auxin known to stimulate root formation in plant tissue cultures. To evaluate its effectiveness in *Thymus bovei*, different concentrations of IAA were added to the MS medium. The highest rooting response was observed at 1.0 mg l^{-1} IAA, resulting in an average of 3.5 roots per explant and a root length of 0.32 cm (Table 7, Figure 5). Although IAA positively influenced rooting, its effect was less pronounced compared to IBA under similar conditions. Comparable results were reported in *Origanum elongatum* (Bonnet) Emb. & Maire by Benkaddour et al. (2022), where IAA promoted adventitious root formation. Furthermore, Sagharyan et al. (2021) demonstrated that increasing IAA concentrations enhanced rooting in *Nepeta binaloudensis* Jamzad, with the highest rooting observed at 1.5 mg l^{-1}. These findings suggest that while IAA facilitates root development in *T. bovei*, it is less effective than IBA, and optimal concentrations vary among species.

3.8 CALLUS SUCCESSFULLY INDUCED USING 2,4-D BUT NOT NAA: 2,4-D AT 2.0 MG **L**$^{-1}$ YIELDS THE HIGHEST CALLUS BIOMASS

Callus induction is a crucial step in plant tissue culture for regeneration and secondary metabolite production. To determine the optimal auxin concentration for callus formation in *Thymus bovei*, different levels of 2,4-dichlorophenoxyacetic acid (2,4-D) were tested under light conditions. The highest callus biomass was obtained with 2.0 mg l^{-1} 2,4-D, producing a fresh mass of 0.601 g, which was significantly greater than the control (Table 8, Figure 6). This result confirms the positive role of 2,4-D in promoting callus growth, consistent with previous findings in *Thymus persicus* (Ronniger ex Rech. f.) Jalas reported by Bakhtia et al. (2016). Similar callus induction effects of 2,4-D have been documented in several medicinal plants such as *Achyranthes aspera* L: (Sen et al., 2014), *Vitex negundo* L. (Choudhury et al., 2011), *Aquilaria agallocha* Roxb. (Debnath, 2013), and *Centella asiatica* (L.) Urban (Biradar, 2017). Additionally, Suhartanto et al. (2022) observed that callus fresh mass was higher in cultures grown in the dark (115.1 mg) compared to those grown under light (96.3 mg), highlighting the influence of light conditions on cal-

Table 8: Effect of 2,4-D level on callus induction of *Thymus bovei* after six weeks' growth period.

2,4-D (mg l^{-1})	Dark		Light	
	% of callusing*	Fresh mass (g)	% of callusing	Fresh mass (g)
0.0	0 c	0 dx	0 d	0 d
1.0	100 ± 0 a	0.240 ± 0.01 b	75 ± 15 b	0.343 ± 0.1 b
2.0	100 ± 0 a	0.343 ± 0.01 a	100 ± 0 a	0.601 ± 0.14 a
3.0	50 ± 5 b	0.113 ± 0.02 c	62.5 ± 10 c	0.184 ± 0.0 c

% Callusing = (Number of explants that produced callus/Total number of explants) × 100
[x]The values presented represent the means ± standard error. N = 10

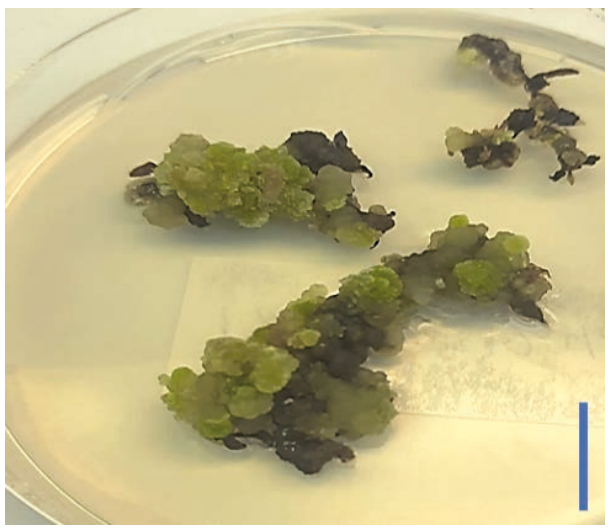

Figure 6: Callus induction from leaf discs on 2,4-D (2.0 mg l^{-1}) under light. The bar represents 1.0 cm.

lus development. Overall, 2,4-D at 2.0 mg l^{-1} under light is effective for inducing substantial callus biomass in *T. bovei*.

3.9 NAA FAILS TO INDUCE CALLUS FORMATION IN LIGHT CONDITIONS

Callus induction is influenced by both plant growth regulators and environmental factors such as light. In this study, no callus formation was observed at any concentration of naphthalene acetic acid (NAA) under light conditions. However, under dark conditions, callus developed successfully, with the highest fresh massof 1.124 g recorded at 3.0 mg l^{-1} NAA (Table 9, Figure 7). This indicates that the effect of NAA on callus induction in *Thymus bovei* is dependent on light exposure. Supporting these findings, Niloofar et al. (2020) reported increased callus production in *Salvia tebesana* Bunge when NAA was added to MS media. Similarly, Bahrames (2005) observed enhanced callusing in *Trigonella corniculata* (L.) L. leaf explants with NAA treatment. These results highlight the importance of optimizing both hormonal and environmental conditions to achieve efficient callus induction.

3.10 ACCLIMATIZED PLANTS SHOW HIGH SURVIVAL RATES UNDER GREENHOUSE CONDITIONS

Successful acclimatization is a crucial step for the transition of in vitro cultured plants to soil conditions. In this study, *Thymus bovei* plantlets were efficiently acclimatized, achieving a high survival rate of 90% for plants treated with 1.0 mg l^{-1} IBA and IAA (Figure 8). Moreover,

Table 9: The effect of 1-naphthalene-acetic (NAA) level on callus induction of *Thymus bovei* after six weeks growth period.

NAA. (mg l^{-1})	Dark		Light	
	% of callusing*	Fresh mass (g)	% of callusing	Fresh mass (g)
0.0	0	0	0	0
1.0	100 ± 0	0.675 ± 0.24 b[x]	0	0
2.0	100 ± 0	0.655 ± 0.11 b	0	0
3.0	100 ± 0	1.124 ± 0.24 a	0	0

% Callusing = (Number of explants that produced callus/Total number of explants) × 100
[x]The values presented represent the means ± standard error. N = 10

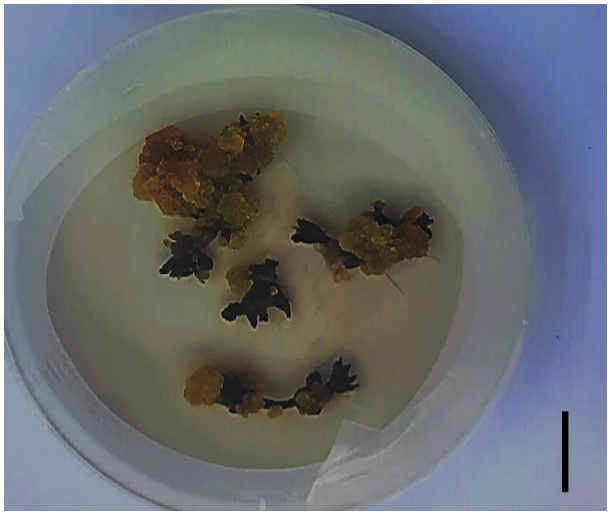

Figure 7: Callus induction from shoot tips on NAA (3.0 mg l-*1*) of *Thymus bovei*. The bar represents 1.0 cm.

Figure 8: Acclimatization of *in vitro* grown *Thymus bovei.* Blue bar represents 3.0 cm.

these plantlets exhibited robust health and vigor under greenhouse conditions. These results are consistent with previous findings by Sarropoulou and Maloupa (2019), who reported successful acclimatization of other Lamiaceae tissue-cultured plants such as *Satureja thymbra* L. Similarly, Teshome and Soromessa (2015) observed excellent ex vitro adaptation and survival rates between 88 % and 96 % in plantlets of various *Satureja* species, including *S. abyssinica*. These findings emphasize the effectiveness of the acclimatization protocols used and suggest promising potential for large-scale propagation of *T. bovei*.

4 CONCLUSIONS

T. bovei was successful multiplicated *in vitro* for the first time in Jordan. MS medium with 2.5 mg l^{-1} KIN and 0.5 mg l^{-1} GA_3 was reported to be the most effective for generating new microshoots. Meanwhile, rooting was the most successful in shoot tips treated with 1.0 mg l^{-1} IBA. Callus formation occurred under both light and dark conditions in media supplemented with 2.0 mg l^{-1} 2,4-D. However, when NAA was used, callus induction was observed only under dark conditions. Meanwhile, most plants were successfully acclimatized after being transferred to greenhouse conditions. However, further research is required on *Thymus bovei* Benth to ensure its long-term preservation and to ascertain the active components present in its essential oils and volatile substances, particularly due to their significance in combating microbial infections and cancerous diseases (Jaradat et al., 2016; Hassan et al., 2018)

Conflicts of interest
None.

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