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ARTICLE

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In vitro callus induction and micropropagation of *Thymus persicus* (Lamiaceae), an endangered medicinal plant

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Abstract – *This is the first attempt towards an efficient regeneration protocol for an endangered and valuable medicinal plant,* Thymus persicus *using* in vitro *callus induction and indirect organogenesis. Callus induction was performed on MS medium supplemented with different concentrations of NAA and 2,4-D, alone or in combination with BAP and KN. Maximum callus induction (100%) was achieved from internode explants cultured on MS medium fortified with 2.0 mg L^{-1} NAA and 0.5 mg L^{-1} KN. The highest frequency of shoot multiplication (96%) was observed with 2.0 mg L^{-1} BAP+1.0 mg L^{-1} NAA. The maximum number of rootlets (16.6 ± 1.4) was induced on half-strength MS medium with 0.5 and 1.0 mg L^{-1} IBA. Rooted plantlets were then successfully grown and acclimatized in the greenhouse with a 70-85% survival rate. The benefits of the protocol described here include all-year-round application, germplasm conservation, suitability for commercial production and also for the biotechnological production of pentacyclic triterpenoids.*

Key words: *Callus culture, regeneration, organogenesis, pentacyclic triterpenoids.*

INTRODUCTION

The genus *Thymus* L. (Lamiaceae) comprises three hundred species of herbaceous annuals and perennials widely distributed throughout the Old World (Morales 2002). *Thymus* species serve as a natural source of monoterpene-phenolic oils, oleoresins, and fresh and dried herbs (Lawrence and Tucker 2002), and have been used for many centuries in traditional medicine (Stahl-Biskup 2002) due to their antiseptic, carminative, antimicrobial, antiviral and antioxidative properties (Reddy et al. 2014). This genus is represented in the flora of Iran by fourteen species, four of which, including *Thymus persicus* (Ronniger ex Rech. f.) Jalas, are endemic (Jalas 1982). *T. persicus* as a cross-pollination plant is restricted to some regions of northwest Iran (Mozaffarian 1996). The plant's aerial part is interesting as a source of pentacyclic triterpenoids (PTs) *i.e.* betulinic acid (BA), oleanolic acid (OA) and ursolic acid (UA) derived from a squalene precursor (Bakhtiar et al. 2014). BA, OA and UA are highly valuable compounds because of their wide spectrum of biological activities, such as anti-inflammatory, hepatoprotective, antitumor, anti-HIV, antimicrobial, antifungal, anti-ulcer, gastroprotective, hypoglycemic, and antihyperlipidemic (Liu et al. 2012, Chudzik et al. 2015).

Owing to a narrow range of distribution, a low propagation rate in nature, land-use disturbances, mine over-exploitation, and over-collection of wild plants for medicinal purposes (Figure 1), *T. persicus* is now almost extinct and is listed as an extremely vulnerable species in Iran (Jalili and Jamzad 1999). There is an obvious need to develop an efficient regeneration system for effective conservation and rapid multiplication in order to replenish highly impoverished populations.

In vitro propagation (IVP) of endangered plants can offer considerable benefits, including rapid cultivation of species that have limited reproductive capacity and exist in threatened habitats (Fay 1992). IVP methods are also essential components of plant genetic resource management and are becoming increasingly important for the conservation of rare and endangered plant species (Almeida et al. 2005, Sidhu 2010). These techniques also facilitate the application of genetic manipulation procedures (Ueno et al. 1996) and long-term storage (Hawkes et al. 2000). In addition, indirect IVP through

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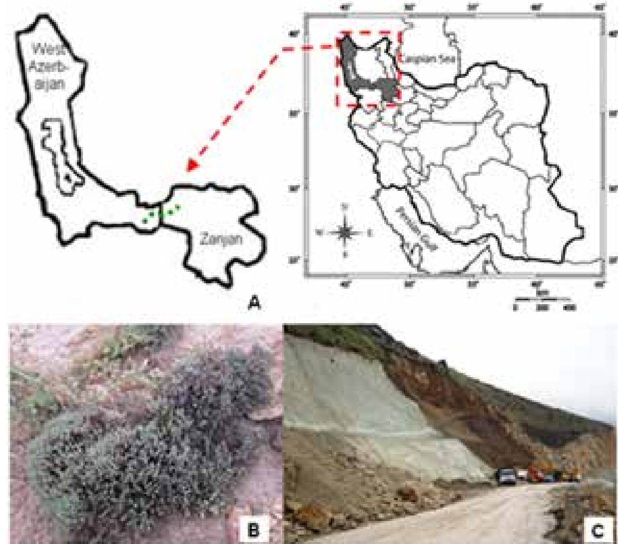

Figure 1. *Thymus persicus*: **A.** Distribution map; **B.** Wild population; **C.** Mine exploitation in the habitat of the plant.

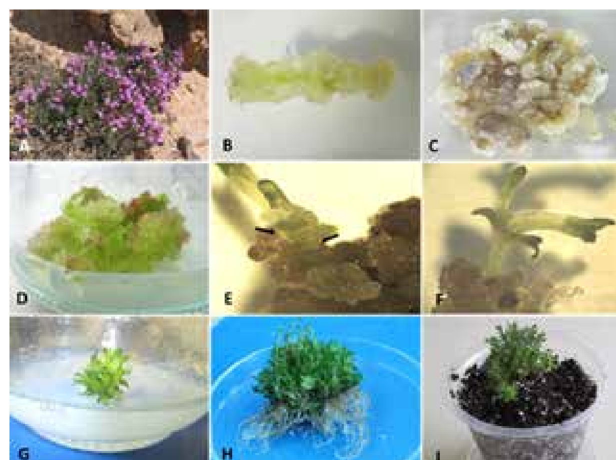

Figure 2. *In vitro* propagation of *Thymus persicus*: **A.** Wild plant; **B.** Internode explant after 21 days on a medium supplemented with 2.0 mg L^{-1} NAA + 0.5 mg L^{-1} KN; **C.** Callus induction after 30 days in the dark; **D.** 3-Days-old callus in light; **E, F.** Shoot formation from callus after incubation for 3 weeks on a medium supplemented with 2.0 mg L^{-1} BAP+ 0.5 mg L^{-1} NAA; **G.** Number of shoots after incubation for 4 weeks on medium supplemented with 2.0 mg L^{-1} BAP + 1.0 mg L^{-1} NAA; **H.** Root formation with 1.0 mg L^{-1} BA; **I.** Transplanted plant in pots.

callus culture is considered to be the most efficient method for crop improvement by the production of somaclonal and gametoclonal variants. This technology has vast potential to produce superior quality plants and allows the isolation of useful variants from well-adapted high-yielding genotypes with better disease resistance and stress tolerance (Brown and Thorpe 1995). Certain types of callus cultures give rise to clones that have inheritable characteristics different from those of parent plants due to the occurrence of somaclonal variability (George 1993), which can lead to the development of commercially important improved varieties (Lee and Chen 2014). IVP protocols have already been established for *Thymus* species, such as *T. vulgaris* and *T. longicaulis* (Ozudogru et al. 2011), and *T. lotocephalus* (Coelho et al. 2012). Recently, micropropagation of *T. persicus* via direct organogenesis has been reported from our laboratory (Bakhtiar et al. 2014). The present work reports an efficient protocol for achieving high-frequency shoot induction and plant regeneration, starting from a callus culture of *T. persicus*, and for the reintroduction of the produced plants into their natural habitat.

MATERIAL AND METHODS

Plant material and sterilization procedure

Mother plants of *T. persicus* were collected from wild populations in the village of Baderloo (lat 36^0 28′ N, long 47^0 13′ E, and alt 2,500 m asl), Takab, in northwest Iran (Figure 2 A). Based on Bakhtiar et al. (2014), healthy plants were separated from the mother plant and washed with running water for 30 min 2-3 times before receiving a pre-treatment with liquid detergent for 30-40 min. Washed explants were immersed in 70% ethanol for 1 min, followed by washing with sterile distilled water, and then soaked in 0.1% sodium hypochlorite (NaOCl) containing a few drops of Tween 20, before being rinsed for 10 min with sterile double distilled water.

Culture conditions and callus induction

Leaf and intermode explants were excised from 1-year-old *in vitro T. persicus* plantlets (Bakhtiar et al. 2014), and were then sectioned into 1-2 cm-long segments. Callus cultures were induced on MS medium (Murashige and Skoog 1962) supplemented with 3% sucrose, 0.8% agar (Merck, Darmstadt, Germany) and different concentrations of auxins 1-naphthalene-acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D), alone or in combination with cytokinins 6-benzylaminopurine (BAP) and kinetin (KN) (Table 1). Callus induction took place in 250 mL glass jars, containing 40 ml of medium, sealed with plastic caps. Cultures were incubated in the dark for 7 days and then incubated under a 16-h photoperiod provided for 30 days by cool-white fluorescent lamps (Philips, 58 W, Holland) at a photon flux density of 40 µmol m^{-2} s^{-1} at 25 ± 2 °C. Each glass jar was considered as an experimental unit and the experiment was conducted at least thrice with 10 replicates per treatment. The cultures were subcultured

Table 1. Effect of auxin and cytokinin type and concentration on callus induction from leaf and internode explants of *Thymus persicus*

Plant growth regulator	Concentration (mg L^{-1})	Leaf		Internode	
		Callus induction (%)	Color and Texture	Callus induction (%)	Color and Texture
Control	0.0	00±0.0	off	12±0.0^{gh}	Brown, compact, hard
2,4-D	1.0	20±1.4^{ef}	Brown, compact, hard	33±1.0^{f}	Brown, compact, hard
	2.0	10±0.0^{fg}	Brown, compact, hard	24±0.9^{fg}	Brown, compact, hard
	3.0	15±0.9^{f}	Dark brown, compact, hard	30±1.0^{f}	Dark brown, compact, hard
NAA	1.0	10±0.7^{fg}	Light brown, compact, hard	12±1.3^{gh}	Light brown, compact, hard
	2.0	40±0.3^{d}	Light brown, compact, hard	57±0.5^{de}	Light brown, compact, hard
	3.0	33±0.4^{e}	Light brown, compact, hard	23±1.2^{fg}	Light brown, compact, hard
2,4-D + BAP	1.0+0.5	96±1.0^{a}	Light brown, friable, wet	98±0.6^{ab}	Light brown, friable, wet
	2.0+0.5	52±0.5^{cd}	Brown, friable, wet	81±0.3^{c}	Brown, friable, wet
	2.0+1.0	21±0.6^{ef}	Dark brown, wet	30±0.7^{f}	Dark brown, wet
2,4-D + KN	1.0+0.5	58±1.0^{c}	Whitish yellow, compact	65±1.1^{d}	Whitish yellow, loose
	2.0+0.5	17±0.2^{f}	Light green, compact	77±0.9^{cd}	Yellow green, compact
	2.0+1.0	85±0.9^{b}	Green, compact	90±0.3^{b}	Light green, compact
NAA + BAP	1.0+0.5	42±0.0^{d}	Light green, compact	50±0.4^{e}	Whitish green, friable, soft
	2.0+0.5	10±0.8^{fg}	Green, compact	10±1.6^{h}	Light green, friable, soft
	2.0+1.0	23±1.9^{ef}	Dark green, compact	20±1.0^{g}	Whitish green, compact
NAA + KN	1.0+0.5	10±0.0^{fg}	White, friable, soft	10±0.7^{h}	Light green, friable, soft
	2.0+0.5	90±0.4^{ab}	White, friable, soft	100±0.0^{a}	White, friable, soft
	2.0+1.0	30±1.1^{e}	Light green, compact	20±0.6^{g}	Light brown, loose

Each value represents Mean±SE. Mean values followed by the same letter are not significantly different at $P<0.05$ (Least Significant Difference Test). Results recorded after 3 weeks of culture.

at regular intervals of 21 days on fresh MS medium. The induction rate (%) and nature of calli (color and texture) were recorded day 35 of induction.

Shoot multiplication and root induction

1-month-old white, friable and soft calli were subcultured on MS media containing different concentrations of BAP, KN, NAA and indole-3-butyric acid (IBA) (Table 2) to induce multiple shoots. The shoot multiplication took place in 250 ml glass jars, containing 50 ml of medium, sealed with plastic caps. The effects of different plant growth regulators (PGRs) on multiple shoot induction and shoot elongation were periodically recorded after 4 weeks. Each glass jar was considered as an experimental unit and the experiment was conducted at least thrice with 5 replicates per treatment. The number of shoots per callus and the rate of shoot induction (%) were counted at day 35 of regeneration (Table 2). Regenerated shoots (1-1.5 cm) were then transferred to MS medium containing 2.0 mg L^{-1} BAP+1.0 mg L^{-1} NAA to elongate shoots for 30 days. The healthy elongated shoots (2-2.5 cm) were then transferred to half-strength MS basal medium supplemented with different concentrations of IBA and NAA (Table 3) to root for four weeks, and then the rooting rate was counted.

Acclimatization

Plantlets with well-developed roots (average root length exceeding 10 mm) were removed from the culture medium and washed gently with distilled water to remove the adhering medium. Acclimatization of plantlets was carried out according to the method recently described by Bakhtiar et al. (2014).

Statistics

All experiments were conducted under controlled conditions with three replications. The statistical analysis was performed using one-way analysis of variance (*ANOVA*) and the mean values of different treatments were compared by the least significant difference (LSD) test at $P \leq 0.05$.

RESULTS AND DISCUSSION

Callus induction was observed on MS medium supplemented with different concentrations of 2,4-D and NAA, alone or in combination with BAP and KN (Table 1). Callus initiation was achieved from the explants within 25-30 days of inoculation (Figure 2B). Both types of selected explants gave the maximum percentage of callusing, which confirms previous studies on various plants including *Mentha spicata* (Poovaiah et al. 2006), *Thymus hyemalis* (Nordine et al. 2014), *Lavandula angustifolia* (Machado et al. 2014),

and *Artemisia pallens* (Nathar and Yatoo 2014). All types of media produced callus on both explants except PGRs-free medium (control), which did not produce callus on leaf explants. Depending on the concentration and combination of PGRs, the frequency of callus formation and nature of calli (color and texture) varied widely. Initially, leaf folding and nodal bulging was observed. The callus color and texture ranged from white to dark, and soft to compact-hard (Table 1). At concentrations 1.0 and 2.0 mg L^{-1} 2,4-D, both leaf and internodal explants produced brown calli, whereas at a higher concentration (3.0 mg L^{-1}), compact dark brown calli were formed. In MS media supplemented with NAA, explants developed light brown calli. Our results showed that the use of 1.0 and 2.0 mg L^{-1} NAA in combination with 0.5 mg L^{-1} BAP in induction medium induces white friable calli in internodal explants, whereas NAA in combination with 0.5 mg L^{-1} KN induces white friable calli in both leaf and internodal explants. Alternatively, 1.0 mg L^{-1} 2,4-D combined with 0.5 mg L^{-1} BAP also induced friable calli that were generally light brown (Table 1). Maximum callus induction (100%) was achieved from internode explants cultured on MS medium fortified with 2.0 mg L^{-1} NAA and 0.5 mg L^{-1} KN (Figure 2C) followed by 1.0 mg L^{-1} 2,4-D and 0.5 mg L^{-1} BAP (98%).

The calli generated from different callus induction media were transferred to regeneration media containing different concentrations of BAP and KN, alone or in combination with NAA and IBA (Figure 2D-2F). The percentage of regeneration and number of shoots varied. The response of calli to the different concentrations of PGRs used for shoot formation is represented in Table 2. Various concentrations of BAP in combination with NAA significantly influenced the formation of shoots. The highest rate of regeneration was obtained from calli cultured on MS medium supplemented with 2.0 mg L^{-1} BAP and 1.0 mg L^{-1} NAA, which was therefore selected as the optimal shoot multiplication medium for further experiments. This enabled a 96% shoot proliferation and produced an average of 6.8 ± 1.0 healthy shoots longer than 0.5 cm per regenerating callus (Table 2, Figure 2G). Halving the BAP and NAA content produced only a

Table 2. Effect of auxin and cytokinin type and concentration on shoot induction from callus culture of *Thymus persicus*

Plant growth regulator	Concentration (mg L^{-1})	Regeneration (%)	Mean number of shoots (no. ± SE)	Mean shoot length (cm ± SE)
Control	0.0	78^{c}	1.80 ± 0.3^{b}	0.49 ± 0.05^{ab}
BAP	0.5	56^{f}	0.80 ± 0.5^{b}	0.30 ± 0.03^{b}
	1.0	60^{e}	1.50 ± 0.2^{b}	0.40 ± 0.07^{ab}
	2.0	80^{bc}	1.70 ± 0.4^{b}	0.22 ± 0.04^{b}
	3.0	64^{d}	0.80 ± 0.3^{b}	0.29 ± 0.01^{b}
KN	0.5	75^{cd}	1.5 ± 0.6^{b}	0.20 ± 0.11^{b}
	1.0	78^{c}	1.0 ± 0.1^{b}	0.43 ± 0.01^{ab}
	2.0	80^{bc}	0.9 ± 0.7^{b}	0.50 ± 0.12^{a}
	3.0	75^{cd}	1.1 ± 0.9^{b}	0.32 ± 0.04^{b}
BAP + NAA	1.0+0.5	94^{ab}	2.3 ± 0.3^{ab}	0.87 ± 0.01^{a}
	2.0+0.5	78^{c}	3.4 ± 0.5^{ab}	0.70 ± 0.01^{a}
	2.0+1.0	96^{a}	6.8 ± 1.0^{a}	0.57 ± 0.01^{a}
	3.0+1.0	88^{b}	3.9 ± 0.2^{ab}	0.30 ± 0.07^{ab}
KN + NAA	1.0+0.5	92^{ab}	1.9 ± 0.5^{ab}	0.64 ± 0.07^{a}
	2.0+0.5	80^{bc}	1.7 ± 0.7^{b}	0.54 ± 0.01^{a}
	2.0+1.0	64^{d}	2.0 ± 0.4^{ab}	0.40 ± 0.03^{ab}
	3.0+1.0	50^{fg}	2.7 ± 0.1^{ab}	0.35 ± 0.03^{ab}
BAP + IBA	1.0+0.5	40^{g}	2.8 ± 0.7^{ab}	0.39 ± 0.11^{ab}
	2.0+0.5	77^{c}	1.3 ± 0.3^{b}	0.40 ± 0.04^{ab}
	2.0+1.0	80^{bc}	5.8 ± 0.6^{a}	0.47 ± 0.06^{ab}
	3.0+1.0	57^{ef}	2.2 ± 0.5^{ab}	0.37 ± 0.03^{ab}
KN + IBA	1.0+0.5	30^{h}	2.1 ± 0.3^{ab}	0.25 ± 0.01^{b}
	2.0+0.5	80^{bc}	2.1 ± 0.9^{ab}	0.30 ± 0.10^{b}
	2.0+1.0	78^{c}	1.3 ± 0.1^{b}	0.31 ± 0.08^{b}
	3.0+1.0	56^{f}	2.0 ± 0.3^{ab}	0.22 ± 0.03^{b}

Each value represents Mean±SE. Mean values followed by the same letter are not significantly different at $P<0.05$ (Least Significant Difference Test). Results recorded after 3 weeks of culture.

slight decrease in shoot proliferation percentage (94%). Supplementing the proliferation medium with KN resulted in a less satisfactory shoot proliferation than with BAP. The highest regeneration rates were only 92% when the medium contained 1.0 mg L^{-1} KN and 0.5 mg L^{-1} NAA. Different concentrations of KN reduced shoot number and length, and shoot formation varied between 30-96%. Shoot formation from calli has been previously achieved by cytokinin or auxin supplementation or by varying the mineral concentration in the medium (Kour et al. 2014). The immense activity of BAP compared to other cytokinins is reported in many plants, including *Salvia officinalis* (Tawfik and Mohamed 2007), *Thymus bleicherianus* (Aicha and Abdelmalek 2014), and *Mandevilla guanabarica* (Zorat Cordeiro et al. 2014). As shown in Table 2, calli cultured on PGR-free MS medium exhibited a high shoot formation rate (78%). It can be concluded that the calli were habituated to the PGRs used for callus induction, especially BAP. These results are in agreement with those obtained by Bennici and Bruschi (1999) for *Nicotiana bigelovii* var. *bigelovii*, who found that some habituated callus lines exhibit the capacity to regenerate normal shoots for a period of several months.

Healthy elongated shoots were excised and placed on half-strength MS basal medium supplemented with different concentrations (0.1, 0.5, 1.0, 1.5 and 2.0 mg L^{-1}) of IBA and NAA for the induction of roots. The effect of auxins on both root induction and root length was examined after 4 weeks of culture, and IBA was found to be the most effective for root induction (Table 3). High frequency rooting (90%) and a mean root number of 4.8 ± 0.3 with a length of 1.6 ± 0.03 cm were obtained on the medium containing 0.5 and 1.0 mg L^{-1} IBA, respectively. (Figure 2H). Increasing the IBA concentration from 1.0 to 2.0 mg L^{-1} reduced the number of roots per shoot. Increasing the concentration of NAA was also unfavorable for adventitious root formation. Additionally, 65% rooting and a mean root number of 3.3±0.4 with a length of 0.6 ± 0.04 cm were obtained on half-strength MS medium without any PGRs. Similar to our observation, Nathar and Yatoo (2014) found a lower salt concentration to be effective for *in vitro* root induction. High efficiency of IBA for *in vitro* root induction compared to other auxins has been earlier reported (Bouhouche and Ksiksi 2007). However, root induction with a medium containing a higher concentration (1.0 mg L^{-1}) of IBA was accompanied by a profuse development of calli, which inhibited further development of roots. This result is consistent with similar findings in root induction of *Thymus* spp. where the percentage of rooting was reduced at a higher concentration of IBA (Olszowska and Furmanowa 1992). Media containing NAA generally produced short thick roots, some of which were callogenic, whilst media including IBA as well as medium without any PGRs (control) produced long thin roots.

Plantlets with more than four roots longer than 10 mm were transferred to *ex vitro* conditions and acclimatized. After hardening in a soil mixture of peat moss and perlite (1:1) for three weeks, regenerated *T. persicus* plantlet survival was over 80%, but the rate then decreased, as some plants died in the 4-5 weeks following their transfer to soil. It was observed that *T. persicus in vitro* grown plants require a very gradual acclimatization to the external environment. Seventy-five percent of the plants transferred to pots survived and resumed growth (Figure 2I). No morphological alterations were observed among regenerated plants grown in the greenhouse. Similar observations have been reported in *Salvia* (Tawfik and Mohamed 2007).

Table 3. Effect of auxin type and concentration in half-strength MS medium on root formation from regenerated shoots of *Thymus persicus*

PGR	Concentration (mg L^{-1})	Root formation (%)	Mean number of roots (no. ± SE)	Mean root length (cm ± SE)	Root morphology
Control	0.0	65^{cd}	3.3 ± 0.4^{ab}	0.6 ± 0.04^{ab}	Thin, long
IBA	0.1	60^{d}	2.7 ± 0.1^{ab}	0.7 ± 0.07^{ab}	Thin, long
	0.5	90^{a}	1.8 ± 0.5^{ab}	1.1 ± 0.10^{ab}	Fragile, long
	1.0	88^{ab}	4.8 ± 0.3^{a}	1.6 ± 0.03^{a}	Fragile, long
	1.5	67^{c}	1.7 ± 0.2^{ab}	0.9 ± 0.04^{ab}	Fragile, long
	2.0	68^{c}	1.1 ± 0.7^{b}	0.2 ± 0.01^{b}	Thick, short, callus at base
NAA	0.1	50^{e}	1.9 ± 0.3^{ab}	0.6 ± 0.05^{ab}	Thin, long
	0.5	47^{ef}	3.8 ± 0.9^{a}	0.5 ± 0.09^{ab}	Thick, short
	1.0	50^{e}	2.6 ± 0.6^{ab}	0.4 ± 0.04^{ab}	Thick, short, callus at base
	1.5	40^{f}	0.5 ± 0.4^{b}	0.3 ± 0.01^{b}	Thick, short, callus at base
	2.0	25^{g}	0.2 ± 0.1^{b}	0.2 ± 0.02^{b}	Thick, short, callus at base

Each value represents Mean±SE. Mean values followed by the same letter are not significantly different at P<0.05 (Least Significant Difference Test). Results recorded after 3 weeks of culture.

CONCLUSION

In conclusion, the effectiveness of callus induction and plant regeneration depends on the type of growth regulators used and explant source. The results of this work showed that *in vitro*-cultured *T. persicus* explants respond differently according to the growth regulators added to the culture medium. Maximum callus induction (100%) was obtained on internode segments cultured on MS medium supplemented with 2 mg L^{-1} of NAA and 0.5 mg L^{-1} of KN. A combination of BAP and NAA as MS medium supplements proved to be an effective PGR combination, with significantly higher shoot induction and multiplication frequencies. The highest number of shoots per explant was obtained on MS medium supplemented with 2 mg L^{-1} BAP and 1.0 mg L^{-1} NAA. Auxin type and concentration significantly influenced the formation of roots, IBA being the most effective. High frequency rooting (88%) and a mean root number of 4.8 $\pm$ 0.3 with a length of 1.6 $\pm$ 0.03 cm were achieved on the medium containing 0.5 mg L^{-1} IBA.

The method described for the first time in the present study provides an efficient reproducible protocol for *in vitro* micropropagation of *T. persicus* as a potent natural source of antitumor pentacyclic triterpenes. This protocol can also be used for the mass production of *T. persicus* plants, thus contributing to the germplasm conservation of this endangered and valuable medicinal species in the wild. The application of the protocol will facilitate research into the enhanced production of antitumor compounds through different biotechnological strategies, such as plant cell, tissue and organ cultures, and large-scale cultivation in bioreactors.

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