In Vitro Propagation and Nuclear DNA Content of *Tripleurospermum Insularum* (Asteraceae) – A Critically Endangered Insular Endemic Species From Turkey

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Abstract

Tripleurospermum insularum Inceer & Hayirlioglu-Ayaz (Asteraceae) is a critically endangered endemic species in Turkey that is face the risk of extinction as a result of the fragmentation of its habitat as well as overgrazing and trampling of its natural population. However, the protocol for micropropagation of this threatened species has not been developed yet. Here, its regeneration ability on MS media supplemented with different plant growth regulators were evaluated using nodal segments. The higher number and length of shoot per explant was achieved with the addition 4.6 μM ZEA and 0.5 μM IAA to the culture medium. Besides, the highest node number of shoot per explant was obtained from MS medium supplemented with 4.6 μM ZEA and 0.5 μM IBA. Flow cytometric analysis also revealed that most of the *in vitro* developed shoots of *T. insularum* possessed similar nuclear DNA content as well as ploidy level as initial material and plants from natural population. *In vitro* rooting of shoots was achieved at 100 % efficiency containing 2.9 μM IAA. Rooted and well-developed plantlets were initially acclimatized under greenhouse conditions and then moved to the botanical garden, where they matured and flowered. Finally, 76% and 74% survivals were achieved during the acclimatization process, respectively. This is the first report of a successfully developed micropropagation protocol of threatened *T. insularum* for its *ex situ* conservation.

Introduction

The overall loss of biodiversity as well as the number of threatened plant species in the world has increased in recent years due to several anthropogenic pressures and/or environmental factors (Corral et al. 2011). Therefore, it is imperative to implement conservation strategies for the conservation and propagation of phylogenetic resources from each geographic region (Assédé et al. 2018; Deplazes-Zemp 2018; Ramírez-Mosqueda et al. 2019).

The current International Union for Conservation of Nature (IUCN) Red List categories and criteria are constructed to assess the threatened status of species or lower taxa on global or sub-global (national or regional) levels as a means of classifying the relative risk of extinction of taxon (Inceer and Aksu Kalmuk 2019). Additionally, IUCN Red List database shows that plant extinctions are dominated by insular species, and the extinction data are associated with lower competitive ability and anthropogenic factors (Gray 2018). Indeed, insular species are exposed to multiple threats, such as invasions, land use change, and global climate change (Caujapé-Castells et al. 2010; Kueffer et al. 2010; Bellard et al. 2013; Harter et al. 2015; Médail 2017). This general trend is particularly alarming in the islands and islets of the Mediterranean Basin, one of the main centers of plant diversity (e.g. Cañadas et al. 2014; Médail 2017; Cogoni et al. 2019).

Ex situ conservation methods have played an important role in the conservation of threatened plants (Coste et al. 2012). In this context, *in vitro* culture techniques have permitted an increasingly important viable system for rapid, true-to-type mass multiplication as well as conservation of threatened endemic and rare species (Piovan et al. 2010; Grigoriadou et al. 2011; Mallón et al. 2011; Wadl et al. 2011; Valletta et al. 2015). Furthermore, the genetic diversity of local ecotypes can be maximized if plants are multiplied from seeds (Benson et al. 2000a; Coste et al. 2012).

Tripleurospermum Sch.Bip. is a small genus of the tribe Anthemideae of the family Asteraceae with ca. 40 species that is mainly distributed in Europe, temperate Asia, North America and North Africa (Oberprieler et al. 2007). *Tripleurospermum insularum* Inceer & Hayirlioglu-Ayaz is an insular endemic species with a highly restricted geographic range in Turkey, and is known from the single population in Gökçeada, one of the Aegean Islands (Inceer and Hayirlioglu-Ayaz 2014). It grows in open places and on rocky slopes in Gökçeada. On the other hand, *T. insularum* has a pharmaceutical potential (Zeljko et al. 2015; Colak et al. 2017), similarly to other endemic taxa of *Tripleurospermum* (Cansaran et al. 2007; Altundag and Ozturk 2011; Han and Bulut 2015; Mohammadi et al. 2016).

According to Inceer and Hayirlioglu-Ayaz (2014), *T. insularum* is classified within CR category of IUCN. The Red List extinction data of this species are associated with lower competitive ability and anthropogenic factors such as overgrazing, trampling and fragmentation. The population is therefore facing extinction in the near future, unless *in situ* and/or *ex situ* conservation strategies are adopted as soon as possible. To our knowledge, no investigations have been carried out on the *in vitro* culture of members of the genus *Tripleurospermum*. The aim of the present study is to develop the efficient *in vitro* micropropagation protocol for the threatened *T. insularum* and to evaluate the genetic fidelity of regenerants in order to *ex situ* conservation of this insular endemic species.

Material And Methods

Plant material and achene germination

The mature achenes of *T. insularum* intended for evaluation as explants were supplied from the seed collection of Dr. Huseyin Inceer deposited at 4°C at the Karadeniz Technical University, Department of Biology, Trabzon (Çanakkale, Gökçeada, 30 m a.s.l. 17 April 2009, Inceer 717).

Initially, the achenes were washed with tap water for 30 min and then treated with 70% (v/v) ethanol for 30 s. After removal of the ethanol, the achenes were disinfected with 3% sodium hypochlorite (NaOCl) for 10 min. Finally, the disinfected achenes were washed with sterile distilled deionized water three times for 15 min, and then cultured in Murashige and Skoog (MS) (Murashige and Skoog 1962) (Duchefa), and Gamborg's B-5 (B5) (Gamborg et al. 1968) (Duchefa) basal media, each containing 4.7 µM kinetin (KIN) (Sigma). At the end of the 30th day, the plantlets were evaluated for germination percentage to determine which of these two main basal media was better suited to *T. insularum*.

Shoot proliferation

In the shoot multiplication studies, nodal segments obtained from seedling shoots after the third subculture were used as an explant. MS basal medium including vitamins and containing 2% (w/v) sucrose (Duchefa) and 0.8% (w/v) phyto agar (Duchefa) was selected as the most suitable medium for shoot proliferation studies. 6-benzylaminopurine (6-BA, 4.4 µM), KIN (4.7 µM), 6-(γ,γ-dimethylallylamino)-purine (2iP, 4.9 µM), thidiazuron (TDZ, 4.5 µM) and zeatin (ZEA, 4.6 µM) in combination with indole-3-butyric acid (IBA), indole-3-acetic acid (IAA) or α-naphthalene acetic acid (NAA) (0.5 µM) were added to the medium to support the basic basal medium and increase the shoot multiplication rate. All plant growth regulators (PGRs) used in this study and supplied by Sigma were filter-sterilized with 0.22-µm filters, except for 6-BA and NAA, and were added to the cooled media after autoclaving. The pH of the media was adjusted to 5.8 with 1 M HCl or 1 M NaOH before autoclaving. All cultures were maintained at 24 ± 2°C under a 16/8 h photoperiod at a photosynthetic flux of 50 µmol m⁻² s⁻¹, provided by cool daylight fluorescent lamps. The subculturing protocol was performed every four weeks. Multiplication rates were calculated by assessing the number of shoots per explant, length of shoots, the number of leaves on each shoot, callus formation, and plant quality (internode length and shoot thickness).

Nuclear DNA content

Flow cytometry was used to check for the stability of nuclear DNA content as well as ploidy level in *in vitro* derived shoots of *T. insularum*. The leaves of 30-day-old seedlings and of shoots multiplied on MS media supplemented with cytokinins (KIN, 2iP, TDZ, 6-BA and ZEA) and auxins (IBA, IAA, and NAA) were used for flow cytometric analysis. Leaf fragments of the sample plant and the standard plant (*Zea mays* L.) were chopped up using a razor blade in 1 mL of woody plant buffer (Loureiro et al. 2007, 0.2 M Tris HCl, 4 mM MgCl₂.6H₂O, 2 mM EDTA, Na₂.2H₂O, 86 mM NaCl, 10 mM K₂S₂O₅, 1% PVP-10, 1% (v/v) Triton X-100, pH 7.5), supplemented with 50 µg mL⁻¹ propidium iodide and 50 µg mL⁻¹ DNase-free RNase, filtered through a 30-µm mesh and stored on ice, in the dark, until measurement. Three independent samples were extracted, filtered and then measured using a BD Accuri™ C6 instrument. Usually 10.000 nuclei per sample were analysed for nuclear DNA content and absolute values (Inceer et al. 2016). DNA content was then calculated from mean values of G1 peaks according to the following formula:

$$\text{Sample 2C DNA content} = \frac{(\text{sample G1 peak mean}) \times (\text{standard 2C DNA content})}{\text{standard G1 peak mean}}$$

Root induction

MS media, each individually supplemented with 2.5 µM IBA, 2.9 µM, IAA and 2.7 µM NAA and without growth regulators, were again selected for rooting the well-proliferated and sufficiently elongated shoots (≥ 20 mm). Four weeks after the transfer of shoots to the root media, the rooting percentage, number of roots per shoot, root length and secondary root number (lateral roots on the longest root) were calculated to evaluate the rooting success. Each experiment was carried out in triplicate.

Acclimatization

After rooting studies, rooted and well-developed plants were selected to determine their survival rates under greenhouse conditions as well as in the botanical garden. The greenhouse humidity and temperature were adjusted to 80-85% and 26 ± 2°C, respectively. After

30 days, the plants were moved to a field in the botanical garden. While 100% peat was employed in the greenhouse, 1:1 (v/v) peat and forest soil was used for transferring the plantlets to the botanical garden.

Statistical analysis

For all germination experiments, five achenes were placed into each Magenta vessel, and four vessels were prepared per treatment. Each treatment was performed in triplicate. Statistical differences between obtained from shoot proliferation, root induction and flow cytometric studies were calculated on Statistical Package for the Social Sciences software (SPSS version 21). Duncan's multiple range test (DMRT, 95% confidence level) from one-way analysis of variance (ANOVA) was used to detect the statistical significance of differences among the mean values in shoot multiplication and root induction. Nuclear DNA data were evaluated using ANOVA and Dunnett's test at $P = 0.05$.

Results

In vitro germination

The first germination data were obtained at the end of days 8 and 10 after inoculation of the achenes in MS and B5 media, respectively. The MS medium had a greater effect than B5 medium, and yielded 8% more achene germination. Fifty-eight percent germination success was obtained in MS medium, compared to 50% in B5 medium. These two culture media exhibited significant differences in terms of numbers of germination achenes, ($P < 0.05$). MS medium was therefore employed in subsequent shoot multiplication studies of *T. insularum*.

Shoot proliferation

In micropropagation studies, the addition of PGRs to the basal media makes a significant contribution to growth development factors such as length of shoots, lateral shoot formation, and shoot thickness. Furthermore, these PGRs added as cytokinin and auxin combinations differ depending on the plant species and basal medium components. *In vitro* shoot proliferation from nodal explants was successfully achieved after 4 weeks. MS media supplemented with ZEA and IAA, ZEA and IBA or cytokinin and auxin-free media were found to be more effective in terms of all the tested parameters in shoot proliferation studies for *T. insularum*.

The maximum shoot number per explant (3.33 ± 0.48) for this species was recorded with MS medium supplemented with $4.6 \mu\text{M}$ ZEA and $0.5 \mu\text{M}$ IAA (Fig. 1a). Besides, TDZ applications gave lower values in terms of shoot numbers, and there were significant differences between these and other applications. MS medium with the same added cytokinin and auxin combination again reached the highest shoot elongation values with 39.68 ± 3.33 mm. Surprisingly, the cytokinin and auxin-free MS medium yielded the same shoot length value as MS medium with added $4.6 \mu\text{M}$ ZEA and $0.5 \mu\text{M}$ IAA (Table 1; Fig. 1a). On the other hand, MS medium containing 6-BA combined with any auxin was found to be unsuitable in terms of shoot length among all the applied cytokinin and auxin combinations. The lowest shoot length (13.48 ± 1.32) for this species was obtained from MS media containing $4.4 \mu\text{M}$ 6-BA and $0.5 \mu\text{M}$ IAA (Table 1). Significant differences were also observed between ZEA as well as control application and other applied cytokinins in terms of shoot length ($P < 0.05$).

In contrast to the mean values for shoot number and shoot length, MS medium supplemented with $4.6 \mu\text{M}$ ZEA and $0.5 \mu\text{M}$ IBA yielded the highest node number value with 10.67 ± 0.76 nodes per explant. An interesting result for this parameter was also obtained from control with 10.46 ± 1.06 nodes per explant (Table 1). On the other hand, there were significant differences between these two applications and the others ($P < 0.05$).

In the present study, evaluation of callus formation has revealed a significant response in all applications except for control and KIN plus IAA applications. Among all the treatments, TDZ in combination with IBA or IAA were more effective than other treatments in callus formation. TDZ in combination with IBA or IAA alone resulted in 100% callus formation. Although ZEA and NAA (54.17%) and 2iP and IAA (50%) combinations also caused significant callus formation, statistically significant differences were observed between TDZ and these two applications (Table 1).

Root induction

The healthy shoots above 20 mm elongation were cultured in MS medium containing individually 2.5 μM IBA, 2.9 μM IAA, and 2.7 μM NAA, or auxin-free for root induction. The culture conditions during root initiation and root growth were the same as the shoot formation conditions. The obtained rooting successes differed according to the tested auxin type at the end of the 4th week. Only IAA application yielded 100% rooting success for all tested plantlets. In contrast, the highest values in terms of the evaluated rooting parameters were obtained from auxin-free and NAA supported MS media.

Although there was little difference between the four tested different media in terms of root formation times, the first root was seen on day 19 in MS medium supplemented with 2.7 μM NAA. Approximately 20 mm of root formation in culture conditions may be sufficient for acclimatization studies (Fig. 1b). However, the seedlings were kept in culture medium for four weeks due to the delayed initial root induction and in order to keep culture times equal to shoot formation times. Depending on the first root formation, the highest root numbers, as well as secondary root numbers, were obtained from this medium with 2.46 ± 0.48 and 17.63 ± 0.91 , respectively (Table 2). The differences between the highest and the lowest root number were shown in Table 2 ($P < 0.05$). An efficient root length was observed in auxin-free medium with 59.1 ± 3.91 mm, compared with other auxin-treated media (Fig. 2). The lowest mean root length with 46.33 ± 3.41 mm among the groups was observed in MS medium supplemented with 2.5 μM IBA. Significant differences were determined in terms of root length values between all the tested media for root induction ($P < 0.05$).

Genetic stability

The results obtained from flow cytometry analysis of propidium iodide-stained nuclei are summarized in Table 1. As shown in Table 1, nuclear DNA content (2C-values) ranges from 5.85 pg in 4.7 μM KIN combined with 0.5 μM IAA to 7.05 pg in 4.7 μM KIN combined with 0.5 μM NAA. There are no significant differences in 2C-values between regenerated plantlets and plants from natural population, except for the regenerated plantlets in MS media supplemented with 4.7 μM KIN and 0.5 μM IBA, 4.6 μM ZEA and 0.5 μM IBA, 4.7 μM KIN and 0.5 μM NAA, 4.4 μM 6-BA and 0.5 μM NAA and 4.9 μM 2iP and 0.5 μM NAA. On the other hand, all regenerated plantlets have the same ploidy level (2x) as plants from natural population.

Ex situ collection

The rooted and well-developed plantlets were initially acclimatized under greenhouse conditions. The plants were healthy, green and grew rapidly as well as some of them also formed buds and then bloomed under greenhouse conditions (Fig. 1c and 1d). The survival frequency was approximately 76% for *T. insularum* under greenhouse conditions (Fig. 3). After 30 days, the plants were moved to a field in the botanical garden. After a month, they developed new leaves, and some also formed buds and then bloomed (Fig. 1e and 1f). The survival frequency was approximately 74% for *T. insularum* under botanical garden conditions. The micropropagated plants were phenotypically indistinguishable from their seed-derived counterparts.

Discussion

In vitro culture of plant tissues has been used in conservation programs for many threatened species and is especially important in small populations (Gonçalves et al. 2010; Mallón et al. 2011). At the same time, during *in vitro* culture of the plant tissues, the explant type, its orientation in the culture medium, and the types and concentrations of plant growth regulators in the medium can play a significant role in controlling the differentiation processes, while the choice of explant varies by species (Coste et al. 2012). In Turkey, *T. insularum*, a critically endangered endemic species, is face the risk of extinction because of several anthropogenic pressures in its habitat and thus, the species is in urgent need of conservation. The present study is the first report of a successfully developed micropropagation protocol for the *in vitro* culture of *T. insularum* using nodal segments in MS media supplemented with cytokinin (6-BA, KIN, 2iP, TDZ and ZEA) and auxin (IAA, IBA and NAA). Additionally, it is noteworthy to study *ex situ* conservation of this species as well as the genus *Tripleurospermum*.

The present results indicate that the rate of *in vitro* achene germination in *T. insularum* were relatively low because of recalcitrant, which is in line with our previous *ex vitro* germination test for chromosome counting (Inceer and Hayırlıoğlu-Ayaz 2014, data not shown). Leyva-Peralta et al. (2019) noted that the low seed germination yields have attributed to the heterogeneous maturation of the seeds in the Asteraceae, resulting in a high proportion of empty seeds. According to Benson (2000b) and Leyva-Peralta et al. (2019), recalcitrance is the inability of plant cells, tissues, and organs to respond to tissue culture. However, the obtained seedlings of *T. insularum* were vigorous and suitable for use as a source of explants.

Different *in vitro* culture media supplemented with PGRs have been used for *in vitro* culture of other members of Asteraceae (Evenor and Reuveni 2004; Mallón et al. 2011; Shinde et al. 2017) with the MS basal medium being the most applied one. Similarly, shoot production of *T. insularum* was enhanced when shoot nodal explants were cultured in MS medium, which proved to be the most adequate medium tested.

The combinations of cytokinins and auxins have been used to induce shoots from different types of explants in many members of the family Asteraceae (Nikam and Shitole 1998; Sivaram and Mukundan 2003; Radhika et al. 2006; Enyew and Feyissa 2019). On the other hand, MS medium with 6-BA and IAA combination was found to yield a better response for shoot production in some members of the tribe Anthemideae of Asteraceae (Echeverrigaray et al. 2000; Evenor and Reuveni 2004). However, in this study, the use of 4.5 μ M TDZ plus 0.5 μ M IBA as well as 4.4 μ M 6-BA plus 0.5 μ M IAA resulted in the lower shoot number and length respectively, indicative that they are not as effective in shoot regeneration for *T. insularum* (Table 1). Among the various growth regulators tested, the best proliferation of shoots was obtained on the MS medium with 4.6 μ M ZEA and 0.5 μ M IAA combination (Table 1). Besides, depending on the using 4.6 μ M ZEA plus 0.5 μ M IAA, there is no statistically difference in nuclear DNA content and ploidy level between *in vitro* and *in vivo* plants, indicative that they maintain their genetic stability during *in vitro* culture. These findings show that MS medium containing 4.6 μ M ZEA plus 0.5 μ M IAA are the most favorable for shoot proliferation of *T. insularum*. The present results are in agreement with the previous report in *Helichrysum italicum* (Roth) G. Don (Asteraceae) (Giovannini et al. 2003).

The present results show that the percentage of the callus formation is very high in MS medium supplemented with 4.5 μ M TDZ and 0.5 μ M IBA as well as 4.5 μ M TDZ and 0.5 μ M IAA (Table 1). Additionally, the use of 4.5 μ M TDZ in combination with 0.5 μ M IBA resulted in the lower shoot number and length, indicative that they are not as effective in shoot regeneration for this species. Similar results are reported from other species of Asteraceae such as *Gerbera jamesonii* H. Bolus ex Hook (Chakrabarty and Datta 2008), and *Stevia rebaudiana* Bertoni (Zayova et al. 2013).

The auxins such as IAA, IBA and NAA in MS media have been used in different concentrations for rooting in the members of the Asteraceae family (Cardoso and Silva 2013; Shinde et al. 2017). Some members of Asteraceae developed *in vitro* roots on medium free of PGRs, while others required the presence of PGRs in medium to form roots (Trejgell et al. 2010, 2018; Opadobe et al. 2017). According to Opadobe et al. (2017), the incidence of root formation on auxin-free medium may be due to the presence of endogenous auxin in regenerated shoots. In *Saussurea obvallata* (DC.) Edgew., the presence of auxin increased numbers of rooted shoots and numbers of roots per shoot (Joshi and Dhar 2003). Perica et al. (2008) reported that the highest rooting percentage (100%) from shoot explants of *Inula verbascifolia* (Willd.) Hausskn was observed on MS medium supplemented with 2.9 μ M IAA. Owies et al. (2004) also noted that MS medium supplemented with 2.7 μ M NAA yielded a better response for root number in another species *Gundelia tournefortii* L. of Asteraceae. In the present study, the rooting has occurred both with and without auxin in all tested media, but the presence of auxin greatly increased the rooting rate compared to auxin-free medium at the end of the 4th week (Table 2). Using 2.9 μ M IAA resulted in the maximum rooting frequency (100%). On the other hand, the maximum root number (2.46 ± 0.48) as well as secondary root number (17.63 ± 0.91) per explant were obtained from MS medium supplemented with 2.9 μ M NAA (Table 2). On the contrary, auxin free MS medium yielded a better response for root length. The present results revealed that IAA and NAA were more effective than IBA in the induction of roots in regenerated shoots of *T. insularum*. Additionally, the incidence of root formation on auxin-free medium may be due to the presence of endogenous auxin in regenerated shoots.

According to (Dolezel et al. 2007), mixoploidy, polyploidy and aneuploidy can occur in *in vitro* plant cultures and are particularly undesirable in regenerants. Compared with the natural environment, *in vitro* culture is more complicated and stressful, which is more likely to cause genetic variation (Bauri et al. 2011; Cui et al. 2019). Therefore, it is necessary to assess the genetic uniformity of the regenerated plants before confirming the success of a micropropagation protocol as well as reintroduction to natural sites or planting in botanic gardens (Slazak et al. 2015). For the last several years, the flow cytometry technique was extensively used to verify the genetic fidelity of micropropagated plants (Bairu et al. 2011; Ochatt et al. 2011; Slazak et al. 2015; Żabicki et al. 2019). For Asteraceae micropropagation protocols, the genetic uniformity of regenerated plants has been estimated by flow cytometry (Sliwinska and Thiem 2007; Mallón et al. 2010). In our study, all regenerated plantlets of *T. insularum* possessed the same ploidy (2x, i.e. diploid) as initial plants and plants from natural populations. Besides, most regenerated plantlets had almost similar nuclear DNA content, while previously published data for this species from natural population were slightly lower (Inceer et al. 2018). These findings indicated genetic uniformity of regenerated plantlets obtained via direct shoot formation on MS media supplemented with PGRs. On the other hand, using KIN plus IBA, ZEA plus IBA, KIN plus NAA, 6-BA plus NAA and 2iP plus NAA yielded higher 2C-values in

regenerated plantlets of *T. insularum*. The significant differences in 2C-values of these regenerated plantlets may represent genetic variations based on mutations in DNA.

Conclusion

This study is the first report of the efficient *in vitro* propagation of threatened insular endemic *T. insularum*. Nodal explants were used as excellent starting material for axillary shoot multiplication. Multiple shoots possessed almost the same nuclear DNA content as native plants, except when they were grown on medium supplemented with KIN plus IBA, ZEA plus IBA, KIN plus NAA, 6-BA plus NAA, and 2iP plus NAA. Additionally, the present results confirm that the proposed protocol guarantees *in vitro* production of *T. insularum* with genetic stability. The plants obtained *in vitro* via direct organogenesis exhibited normal development, were fertile, and seemed to adapt well to the botanical garden. Hence, this protocol may be used in future conservation programs such as *in situ* activities on population restoration and cryopreservation of this species as well as other threatened endemic species of *Tripleurospermum*.

Abbreviations

MS Murashige and Skoog medium

6-BA 6-Benzylaminopurine

KIN Kinetin (6-furfurylaminopurine)

ZEA Zeatin

2iP 6-(γ,γ -Dimethylallylamino)purine

IAA Indole-3-acetic acid

IBA Indole-3-butyric acid

TDZ Thidiazuron (N-phenyl-N'-1,2,3-thiadiazol-5'-ylurea)

NAA α -naphthalene acetic acid

PGR Plant growth regulator

CONT Control

Declarations

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Tables

Table 1 Effects of different cytokinin in the presence of IBA, IAA and NAA (0.5 µM) combination and control group on the shoot proliferation of *T. insularum* as well as nuclear DNA content (pg/2C ± SD) in the leaves of *T. insularum* from native individuals and *in vitro* derived shoots

Cytokinin (μM)			Auxin (μM)						Shoot Number/ Explant	Shoot Length/ Explant (mm)	Node Number/ Explant	Callus Rate (%)	Plants Quality	2C
CONT	KIN	6-BA	2iP	TDZ	ZEA	IBA	IAA	NAA						
0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.50 ± 0.51cd	39.68 ± 2.68a	10.46 ± 1.06a	nd	+	5.75 ± 0.31**
	4.7					0.5			1.96 ± 0.36ef	19.85 ± 1.78ef	7.17 ± 0.70d	31.93 ± 2.39e	+	6.59 ± 0.23*
		4.4				0.5			2.96 ± 0.55b	15.15 ± 1.19g	8.63 ± 0.77c	30.56 ± 2.40e	-	6.32 ± 0.21
			4.9			0.5			2.00 ± 0.66e	31.43 ± 2.74b	7.50 ± 0.72d	27.78 ± 2.41e	+	6.07 ± 0.54
				4.5		0.5			1.33 ± 0.48g	20.51 ± 1.92e	6.04 ± 0.62e	100.00 ± 0.0a	-	6.56 ± 0.32
					4.6	0.5			2.58 ± 0.58cd	31.57 ± 2.33b	10.67 ± 0.76a	30.56 ± 2.40e	+	6.49 ± 0.18*
	4.7						0.5		2.75 ± 0.44bcd	38.48 ± 2.27b	10.00 ± 0.88b	nd	+	5.85 ± 0.27
		4.4					0.5		2.71 ± 0.46bcd	13.48 ± 1.32h	5.38 ± 0.58f	45.83 ± 4.17c	-	6.17 ± 0.43
			4.9				0.5		2.79 ± 0.59bc	26.11 ± 2.41c	5.58 ± 0.72f	50.00 ± 4.17bc	+	6.27 ± 0.19
				4.5			0.5		1.67 ± 0.48f	19.21 ± 1.81f	3.13 ± 0.45h	100.00 ± 0.0a	-	6.36 ± 0.15
					4.6		0.5		3.33 ± 0.48a	39.68 ± 3.33a	8.71 ± 0.69c	13.89 ± 2.41f	+	6.38 ± 0.16
	4.7							0.5	1.96 ± 0.55ef	26.60 ± 2.21c	7.13 ± 0.74d	40.28 ± 4.81d	+	7.05 ± 0.43*
		4.4						0.5	2.42 ± 0.50d	15.58 ± 1.33g	3.79 ± 0.59g	12.50 ± 0.0f	-	6.73 ± 0.15*
			4.9					0.5	1.88 ± 0.45ef	26.44 ± 2.04c	5.21 ± 0.41f	11.11 ± 2.41f	+	6.70 ± 0.22*
				4.5				0.5	2.50 ± 0.51cd	21.93 ± 1.61d	5.25 ± 0.61f	38.89 ± 2.41d	-	6.41 ± 0.32
					4.6			0.5	2.58 ± 0.50cd	21.93 ± 1.95d	5.33 ± 0.48f	54.17 ± 4.17b	+	6.39 ± 0.37

Data were recorded four weeks after the culture and represent a total of three replicates of 24 plants per treatment on MS. Values having the same letter(s) in the same column are not significantly different according to Duncan's multiple range test at $P < 0.05$. nd = Not detected

* $P = 0.05$, ** Native individuals.

Table 2 The effects of different auxin types on *in vitro* rooting of *T. insularum* shoots

PGRs (μM)	Rooting rate (%)	Root Number (No/Plant)	Root Length (mm)	Secondary Root Number (No/Plant)
Control	91.7 $\pm$ 3.6b	1.65 $\pm$ 0.37c	59.1 $\pm$ 3.91a	10.8 $\pm$ 0.64d
2.5 IBA	89.6 $\pm$ 3.6b	1.82 $\pm$ 0.39bc	46.33 $\pm$ 3.41c	13.8 $\pm$ 1.23c
2.9 IAA	100 $\pm$ 0.0a	2.04 $\pm$ 0.33b	52.2 $\pm$ 2.91b	15.08 $\pm$ 0.97b
2.7 NAA	91.7 $\pm$ 3.6b	2.46 $\pm$ 0.48a	52.91 $\pm$ 3.67b	17.63 $\pm$ 0.91a

Data were recorded on the four weeks after the culture and represent a total of three replicates of 16 plants per treatment for root induction.

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

Figures

Figure 1

Micropropagation and ex vitro collections of *T. insularum*. a 4.6/0.5 μM ZEA/IAA, b 2.7 μM NAA on in vitro rooting of *T. insularum* from shoot-bud culture-derived seedlings, c, d Greenhouse conditions, e, f Botanical garden conditions, Scale bar: 5 cm

Figure 2

The effects of different auxin types on in vitro rooting of *T. insularum* shoots

Figure 3

Survival rates of plantlets transferred from different rooting media to the greenhouse conditions