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In vitro PROPAGATION, COLD PRESERVATION AND CRYOPRESERVATION OF *Taxus baccata* L., AN ENDANGERED MEDICINAL AND ORNAMENTAL SHRUB

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ABSTRACT

In vitro propagation, cold preservation, and cryopreservation are three essential approaches to preserve the genetic resources of red-listed plants, including English yew (*Taxus baccata* L.). Different concentrations of plant growth regulators (PGRs) and different pre-treatments of cold preservation and cryopreservation are the prerequisites of these three approaches. Apical bud as explant and Murashige and Skoog (MS) as the culture medium for all three sections of the research, kinetin (Kin) and indole-3-butyric acid (IBA) as PGRs for the micropropagation section, and encapsulation-dehydration as pre-treatment for the sections of cold preservation and cryopreservation were used. The results of the micropropagation section indicated that the highest number of shoots (5.40 per explant) and roots (5.98 per explant) were obtained from the culture of the explants on the media containing 1 mg L^{-1} IBA together with 1 and 2 mg L^{-1} Kin, respectively. The results of the cold preservation section revealed that the highest percentage of survival of germplasms (100%) after storage in the refrigerator was observed in the apical buds pre-treated by dehydration of encapsulated explants with 0.75 M sucrose for two hours, followed by dehydration under a laminar airflow cabinet for two hours. The results of the cryopreservation section demonstrated that the highest percentage of survival of germplasms (100%) after storage in liquid nitrogen was obtained in the apical buds pre-treated by encapsulation-dehydration under a laminar airflow cabinet for two hours. At the acclimatization stage, 100% of the plantlets acclimatized suitably with *ex vitro* conditions.

Key words: English yew, liquid nitrogen, micropropagation, one-step freezing, plant germplasm, slow growth

INTRODUCTION

The common yew or English yew (*Taxus baccata* L.) from the family Taxaceae is a genus of 5 to 10 species of large shrubs or small ornamental trees, evergreen, and conifers. These species can be found sporadically in the forests of the northern temperate countries, from the Philippines and Central America. English yew is cultivated for its leaves and is used as an ornamental plant in hedges and landscaping [Brickell and Zuk 1997, Tumpa et al. 2022]. Yew is used in various industries such as handicrafts (including carving and sculpture due to the having red wood) and floriculture. Taxol substance (a type of alkaloid) of this tree is used to treat some cancers. *Taxus baccata*, commonly known as the European yew, has a limited distribution

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in Iran. It is primarily found in the country's northern parts, particularly in the Alborz Mountains and the forested regions of Guilan, Mazandaran, and Golestan provinces. Due to their moist and cool climate, these areas provide suitable habitats for *T. baccata* [Mahmoodi et al. 2022].

Taxus baccata is propagated by seeds or by cutting and grafting. Its propagation through seed cultivation faces obstacles and problems, such as the non-uniformity of propagated plants and trait segregation. Therefore, *in vitro* propagation is used to produce true-to-type superior clones. *In vitro* propagation of trees and shrubs has been recognized as an essential and suitable approach for large-scale propagation and overcoming the concerns caused by heterogeneous seed production [Nunes et al. 2018, Li et al. 2021]. The commercial application of tissue culture techniques for the propagation of trees and shrubs, especially hard-rooted species, is limited to a few species due to the ability to change high diversity and the survival of plantlets during acclimatization. Types of explants, types of culture media, and types of plant growth regulators (PGRs) have been evaluated for the success of *in vitro* propagation of trees and shrubs [Aroonpong and Chang 2015]. Due to the slow propagation rate of yew under natural conditions, *in vitro* propagation is suitable for the mass production of this endangered plant. Some protocols have been developed for *in vitro* propagation of different yew species [Kishor et al. 2015, Kochkin et al. 2023]. A literature review revealed that the number of published reports on *in vitro* propagation of different yew species is limited. The highest shoot multiplication and root induction in English yew using bud explants were obtained using 2 mg L^{-1} BAP and 8 mg L^{-1} IBA, respectively [Abbasin et al. 2010]. Some other researchers reported similar results [Chang et al. 2001]. The use of auxins (especially IBA and NAA) and cytokinins (especially BA and BAP) in different concentrations, individually or in combination, for shoot multiplication and root production in some medicinal-ornamental trees and shrubs has been reported [Aroonpong and Chang 2015, Kaviani and Negahdar 2017, Adibi Baladeh and Kaviani 2021, Li et al. 2021, Kaviani et al. 2022].

Preservation of plant biodiversity is necessary for plant breeding and genetic engineering programs. In addition, this biological diversity is a source for use in the pharmaceutical, food, and cosmetic health industries. Preservation of plant biodiversity reduces the risk of genetic erosion. Plant genetic diversity is the foundation of plant breeding and a target of conservation efforts [O'Brien et al. 2020]. To conserve the most considerable number of plant genetic resources worldwide for future food security and breeding programs, it is necessary to integrate *in situ* and *ex situ* conservation methods into a comprehensive conservation plan [O'Brien et al. 2020]. The most suitable method of *ex situ* conservation is the preservation of the germplasms *in vitro* (slow growth and cryopreservation). For storing seedless plants, plants with drought-sensitive seeds, and vegetatively propagated plants, cold preservation, and cryopreservation are suitable methods [Panis et al. 2020]. Encapsulation is one of the critical protective pre-treatments against the cold conditions of the refrigerator and the very low temperature of LN in the germplasm storage techniques in ultra-cold conditions.

Plant materials can be preserved for several years in tissue culture conditions with periodic subcultures using slow-growth methods. About 40,000 accessions are maintained by slow-growth methods worldwide. Few reports are available on the slow-growing *in vitro* preservation of uncommon and endangered ornamental plants [Kaviani 2011, Kulus and Zalewska 2014]. It has been reported that the germplasm of various endangered ornamental species, including vanilla [Divakaran et al. 2006] and clove [Benelli et al. 2012, Arda et al. 2016], has been successfully preserved under cold preservation conditions.

Different biochemical and biophysical mechanisms and dehydration and freezing are required by protocols based on biochemical and biophysical mechanisms to prevent or limit damage to plant tissues during cryopreservation [Vendrame et al. 2014]. Numerous reports have been presented on the cryopreservation of some rare and endangered species, such as several cultivars of orchids, *Buxus hyrcana* Pojark., *Fritillaria imperialis*, and *Lilium ledebourii* (Baker) Bioss. grown in Iran [Kaviani et al. 2008, 2009, Kaviani 2010, Kaviani and Negahdar 2017, Negahdar et al. 2019, Asa and Kaviani 2020]. Long-term preservation techniques (cryopreservation in liquid nitrogen, LN) are widely used for various woody plant collections [Panis 2019, Kaviani and Kulus 2022]. Encapsulation-dehydration technique has been widely used in the cryopreservation of some orchids [Khoddamzadeh et al. 2011, Sub-

ramanian et al. 2011, Mohanty et al. 2012, Surenciski et al. 2012]. Cryopreservation by encapsulation-dehydration pre-treatment on *L. ledebourii* seeds with 50% survival success [Kaviani et al. 2010], *F. imperialis* with 74.30% [Seydi et al. 2020], *Centaurium rigualii* node with 70% [González Benito et al. 1997], *B. sempervirens* with 66.30% [Negahdar et al. 2019], *B. hyrcana* with 60% [Kaviani and Negahdar, 2017], apple rootstock with 45–88% [Paul et al. 2000], plum rootstock with 14–76% and pear with 30–82% survival [Hao et al. 2005] have been reported.

A few studies have been done on cold preservation and cryopreservation of ornamental plants, especially those in danger of extinction. Therefore, the purposes of the current research were to evaluate the effect of different concentrations of Kin and IBA on *in vitro* propagation using shoot apical bud explants through direct organogenesis (the first part of the research), as well as cold preservation (slow growth) in a refrigerator (the second part of the research) and cryopreservation in LN (the third part of the research) using pre-treatments of encapsulation-dehydration on English yew.

MATERIALS AND METHODS

In vitro propagation

The experiments were performed in a greenhouse and laboratory of the Hyrcan Agriculture and Biotechnology Research Institute, Amol city, Mazandaran province, the northern part of Iran, in 2021–2022. The geographical coordinates of Amol are as follows: latitude: 36°28'10" N, longitude: 52°21'02" E, and elevation above sea level: 96 m (ca. 315 ft). This research used yew or English yew (*Taxus baccata* L.) as the mother rootstock, and their shoot apical buds were used as explants. The geographical distribution of this species in Iran is exclusive to the forests of northern Iran. The collected specimens were also morphologically compared with the presented images of this species in floras, catalogs, and books [Ghahraman 2000]. The shoots were disinfected to separate their apical buds. The shoots were washed under running tap water with a few drops of dishwashing liquid for 10 minutes and then rinsed utterly. Apical buds were separated from the main shoots. The buds were sterilized with 70% ethanol (v/v) for 2 minutes and then with sodium hypochlorite solution containing 5% available chlorine and 0.05% (v/v) Tween-20 for 10 minutes under a sterile ambient of a clean airflow cabinet. Finally, the sterilized apical buds were rinsed five times with sterile distilled water for 5 minutes each time.

Surface sterilized apical buds were cultured on Murashige and Skoog (MS) culture medium [Murashige and Skoog 1962] enriched with 0, 0.5, 1, and 2 mg L^{-1} of both Kin and IBA, individually or in combination with each other. Sucrose (3%) was added as a carbon source, and agar (0.7% w/v) was added as a solidifying agent. The pH of the media was adjusted to 5.7 ±0.10 using 1 N NaOH or 0.1 N HCl before sterilization. After adjusting the pH, the culture media were autoclaved (Iran Teb Zaeem Co., Iran) at a temperature of 121°C and atmospheric pressure of 15 psi for 20 minutes. The culture media containing the explants were kept in a growth chamber (Noor Sanat, Iran) with a temperature of 20 ±2°C under a 16-hour photoperiod with an approximate photosynthetic current density of 3,700 Lux (50 μmol m^{-2} s^{-1}) provided by white fluorescent lamps (Philips, the Netherlands) at a relative humidity of 75–80%.

The number of shoots, number of nodes, and number of roots were measured after 90 days. Plantlets with well-developed roots were removed from the culture vessels and washed with sterilized distilled water to remove nutrients, and agar adhered to the roots. These plantlets were then planted in pots filled with a mixture of perlite and peat moss in equal proportion. The plantlets planted in pots were covered with thin plastic bags and transferred to a greenhouse with a temperature of 25 ±2°C and a relative humidity of 70–80%. These plantlets were watered regularly and at the required times. The plantlets were gradually exposed to the external environment by removing the plastic bags from the pots.

Cold preservation and cryopreservation

The buds were sterilized with 70% ethanol (v/v) for 2 minutes and sodium hypochlorite solution containing 5% available chlorine and 0.05% (v/v) Tween-20 for 10 minutes. Under the clean airflow hood cabinet (Jal Tajhiz, Iran), 5–10 mm of the ends of the sterilized shoots (apical buds) were separated and used as explants for cold preservation and cryopreservation (Fig. 1).

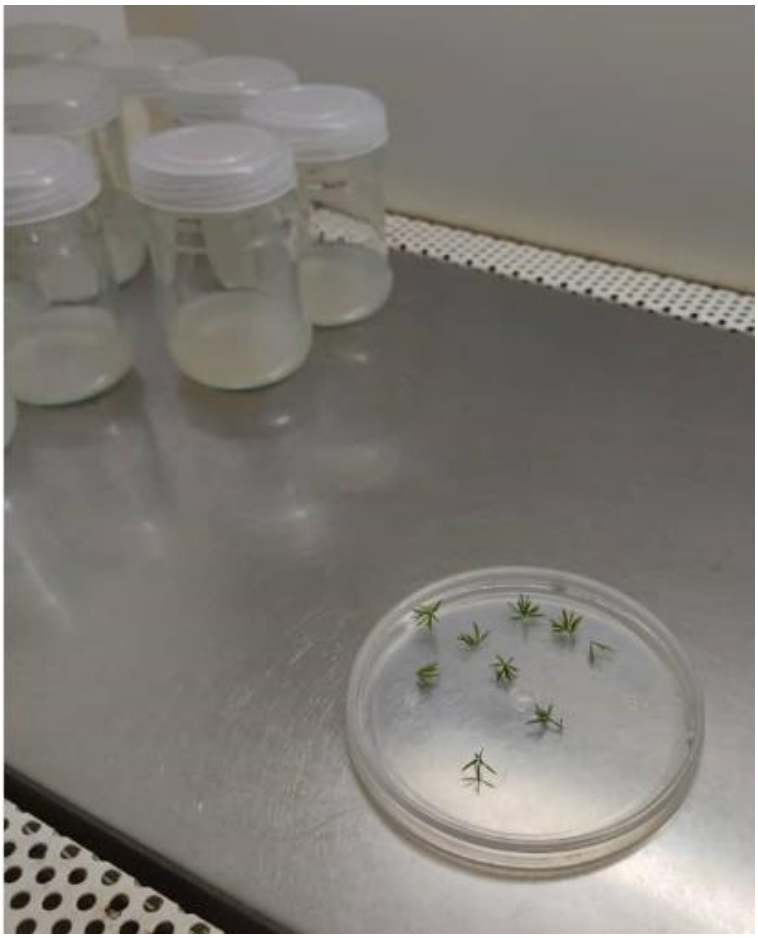

Fig. 1. Apical buds' explants of *T. baccata* L. for use in experiments on the effect of different pre-treatments on the survival of the explants after preservation in cold and ultra-cold conditions

Table 1. Symbols have been used to introduce treatments in the following tables and figures

Treatments symbol	Abbreviations	Treatments
T_1	Control	No treatments
T_2	MS + 0.75 M suc.	Pre-treated with 0.75 M sucrose for 2 h in MS medium
T_3	Deh.	Pre-treated with air-desiccation under a clean airflow cabinet for 2 h
T_4	MS + 0.75 M suc. + Deh.	Pre-treated with 0.75 M sucrose in MS medium for 2 h followed by air-desiccation under a clean airflow cabinet for 2 h
T_5	MS + E	Encapsulated in MS medium containing Na-alginate
T_6	MS + 0.75 M suc. + E	Encapsulated in MS medium containing Na-alginate with 0.75 M sucrose for 2 h
T_7	MS + ED	Encapsulated in MS medium containing Na-alginate followed by air-desiccation under a clean airflow cabinet for 2 h
T_8	MS + 0.75 M suc. + ED	Encapsulated in MS medium containing Na-alginate with 0.75 M sucrose for 2 h followed by air-desiccation under clean airflow cabinet for 2 h

After the pre-treatments presented in Table 1, the apical buds were kept in a refrigerator (LG, South Korea) at a temperature of 4°C for three months for cold preservation with slow growth [Manokari et al. 2021] and for 24 h in an LN tank for cryopreservation with growth arrest [Kaviani and Kulus 2022]. The apical buds were placed inside Petri dishes without a cap under the clean airflow cabinet for 2 hours to dehydrate. To encapsulate the apical buds, they were transferred to an MS culture medium containing 3% sodium alginate (Na-alginate) (Merck, Germany) and remained for one hour. Then, the buds were transferred individually by forceps from this medium to an MS medium containing 100 mM $CaCl_2$ and remained in this medium for one hour. The capsules containing the buds (beads) were then transferred into LN.

For thawing, the cryovials containing cryopreserved shoot tips were rapidly warmed in a water bath at 40 ±2°C for 2 min before being cultured on a recovery medium. Thawing is a critical factor that affects shoot regrowth after LN exposure and ensures the speedy transition from glass to liquid without ice recrystallization within plant cells [Zhang et al. 2023]. These samples and samples kept in a refrigerator (LG, South Korea) were then cultured in regeneration medium (MS basal medium) together with appropriate concentrations of PGRs (obtained in the micropropagation section, i.e., 1 mg L^{-1} Kin together with 1 mg L^{-1} IBA) for proliferation. The cultured samples were kept in the growth chamber with a temperature of 20 ±2°C under a 16-h photoperiod with an approximate photosynthetic current density of 50 ±5 µmol m^{-2} s^{-1} provided by white fluorescent lamps (Philips, the Netherlands) in a relative humidity of 75–80%.

The survival and germination percentage of germplasms (apical buds) were measured in the regeneration medium. If signs of germination (emergence of roots or shoots) are observed in the cultured samples in *in vitro* conditions, it is a sign of their survival, and the survival percentage was considered.

Experimental design and data analysis. The factorial *in vitro* experiment was conducted in a completely randomized block design with four replications. The observations were done every two weeks, and the final result was recorded two months after cultivation in regeneration media. Statistical data analysis was done using Statistical Package for Social Sciences (SPSS) software. To check the significance of the differences between the average data, the least significant difference (LSD) was used at the probability level of 0.01%.

RESULTS

In vitro propagation

The number of shoots. The number of shoots produced by the apical bud explant following the treatments was recorded after about 90 days. The ANOVA results indicated that the interaction effects of IBA and Kin concentrations were significant on the number of shoots ($p < 0.01$). No correlation existed between the number of shoots and the concentration of Kin and IBA (Tab. 2). The treatment containing 1 mg L^{-1} Kin and 1 mg L^{-1} IBA produced the maximum shoots (5.40 per plantlet) compared to the control treatment, which produced the fewest (1.25 per plantlet). The co-application of Kin at 2 mg L^{-1} and IBA at 0.5 mg L^{-1} induced fewer shoots than other treatments (Tab. 2, Fig. 2). Because the most significant number of shoots were observed in the IBA-free treatment, Kin has likely played a more significant impact in boosting the number of shoots than IBA.

Number of nodes. According to ANOVA, the effect of Kin and IBA was significant on the number of nodes ($p < 0.01$). The highest number of nodes (6.67 per plantlet) was observed in apical buds grown on media enriched with 2 mg L^{-1} Kin without IBA, followed by 6.22 nodes per plantlet under 2 mg L^{-1} Kin and 0.5 mg L^{-1} IBA. The lowest number of nodes (1.74 per plantlet) was obtained in explants grown in a culture medium containing Kin and IBA at 0.5 mg L^{-1}, representing a 67% reduction compared to the control (Tab. 2).

Number of roots. IBA and Kin significantly ($p < 0.01$) affected the number of roots. The highest number of roots (5.98 per plantlet) were produced in the culture media containing 1 mg L^{-1} IBA and 0.2 mg L^{-1} Kin, followed by 1 mg L^{-1} IBA and 0.5 mg L^{-1} Kin. However, the lowest root number (1.54 per plantlet) was recorded in the culture medium without the PGRs (Fig. 2). The results indicated that the role of Kin in stimulating root production is almost as crucial as IBA because Kin was present together with IBA in the best treatments to stimulate root production.

Table 2. The effect of different concentrations of IBA and Kin on the number of shoots, nodes, and roots of *T. baccata* L. grown *in vitro* condition

IBA + Kin (mg L^{-1})	Shoot number	Node number	Root number
0.00 + 0.00	1.25^{e} ±0.19	2.53^{b-d} ±0.29	1.54^{e} ±0.48
0.00 + 0.50	1.52^{de} ±0.29	3.04^{bc} ±0.41	2.55^{c-e} ±0.48
0.00 + 1.00	3.75^{b} ±0.48	2.51^{b-d} ±0.29	2.31^{de} ±0.48
0.00 + 2.00	1.51^{de} ±0.25	6.67^{a} ±0.48	3.12^{cd} ±0.25
0.50 + 0.00	1.75^{de} ±0.25	2.10^{cd} ±0.41	1.97^{de} ±0.41
0.50 + 0.50	2.12^{de} ±0.41	1.74^{d} ±0.25	2.65^{c-e} ±0.48
0.50 + 1.00	4.10^{ab} ±0.41	3.01^{bc} ±0.41	3.04^{c-e} ±0.41
0.50 + 2.00	1.75^{de} ±0.25	6.22^{a} ±0.41	3.39^{cd} ±0.64
1.00 + 0.00	1.49^{de} ±0.29	2.01^{cd} ±0.41	3.00^{c-e} ±0.41
1.00 + 0.50	2.10^{de} ±0.41	3.00^{bc} ±0.41	5.30^{b} ±0.41
1.00 + 1.00	5.40^{a} ±0.41	3.55^{b} ±0.25	4.11^{bc} ±0.41
1.00 + 2.00	1.98^{de} ±0.41	3.08^{bc} ±0.41	5.98^{a} ±0.96
2.00 + 0.00	1.75^{de} ±0.48	3.18^{b} ±0.25	2.13^{de} ±0.25
2.00 + 0.50	2.42^{cd} ±0.29	2.89^{bc} ±0.41	2.85^{c-e} ±0.48
2.00 + 1.00	2.19^{c-e} ±0.63	1.81^{d} ±0.25	2.10^{de} ±0.41
2.00 + 2.00	3.17^{bc} ±0.48	2.59^{b-d} ±0.48	3.25^{cd} ±0.25

Values are means ±standard deviations. Different letters in the same column indicate significant differences between means at ($p < 0.01$) based on the LSD test.

Cold preservation

English yew germplasms (apical buds) were encapsulated in an MS culture medium containing Na-alginate and $CaCl_2$. Encapsulated and non-encapsulated germplasms were stored at 4°C in the refrigerator. After storage, they were placed in the culture medium to evaluate the germination potential and growth of apical buds (Figs 3 and 4). Encapsulation was a more suitable pre-treatment than other pre-treatments to increase the resistance of apical buds against the stress of cold conditions. The highest survival percentage of germplasms (100%) after storage in the refrigerator was obtained in the pre-treated apical buds by dehydration from encapsulated explants with 0.75 M sucrose for two hours, followed by dehydration under laminar airflow cabinet for two hours (Tab. 3). The non-pretreated apical buds had lower survival than other pre-treated buds after storage in the refrigerator. Therefore, the lowest percentage of survival of germplasms (71.33%) after storage in the refrigerator was obtained in the apical buds of the control (Tab. 3). The combination of physical and chemical dehydration pre-treatments was more appropriate than any of these two pre-treatments alone, according to a comparison between the pre-treatments of osmotic dehydration with 0.75 M sucrose and physical dehydration in a laminar airflow cabinet (Tab. 3, Figs 4 and 5).

Cryopreservation

The ANOVA showed a significant difference ($p < 0.01$) of cryopreservation pre-treatments on the survival percentage and germination potential of germplasms (apical buds). After storage in LN, the highest survival percentage of germplasms (100%) was obtained in pre-treated buds by dehydrating the encapsulated buds under a laminar airflow cabinet for two hours (Tab. 4). This result shows the more important role of physical dehydration and encapsulation in improving the percentage of survival and germination of germplasms after storage in LN and during cultivation in a culture medium. The non-pretreated apical buds had less survival after storage in LN. Therefore, the lowest survival percentage of germplasms (59.33%) after storage in LN was obtained in control apical buds

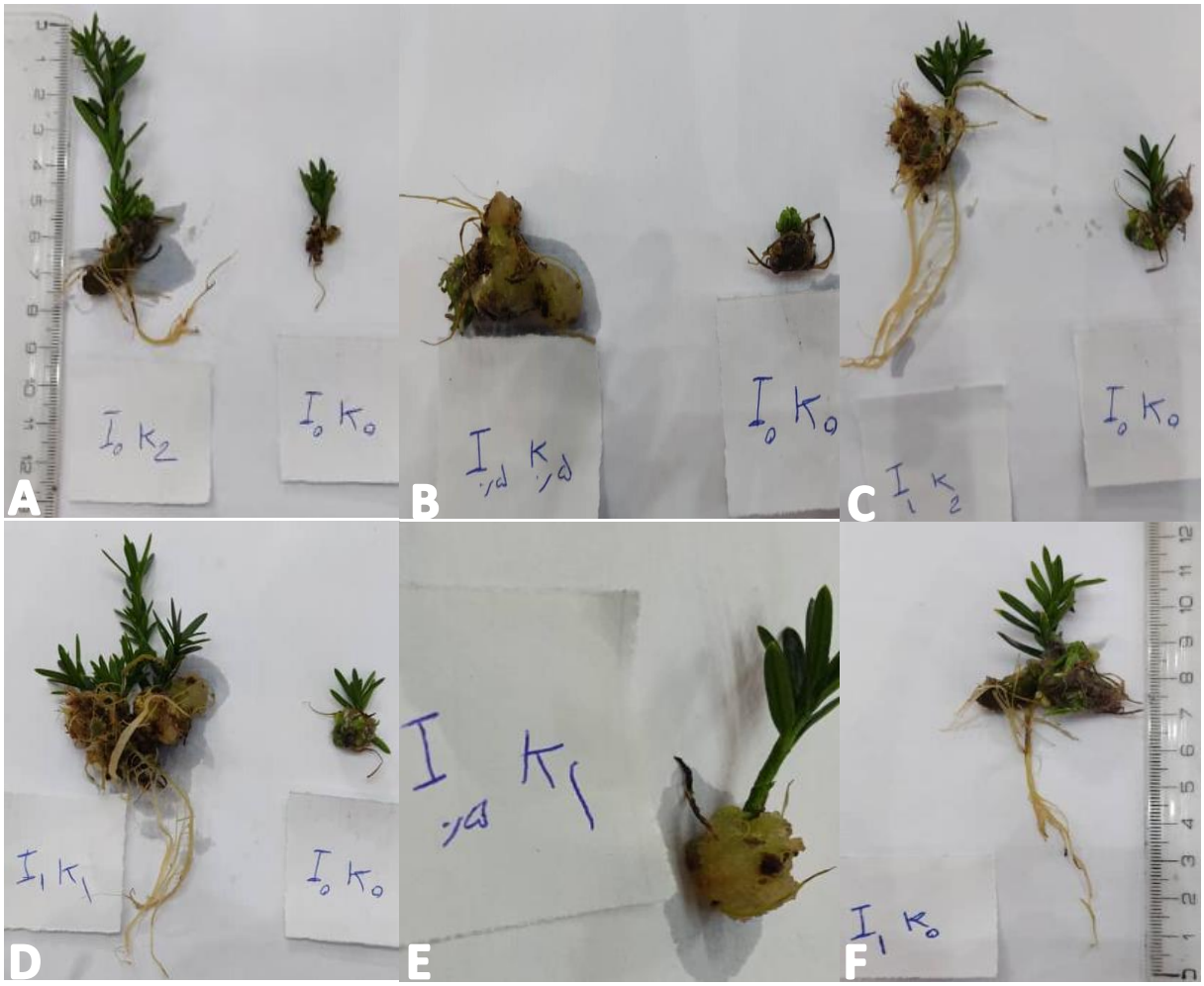

Fig. 2. Micropropagation of English yew (*Taxus baccata* L.) with apical buds. (**A**) Plantlets produced on culture medium containing 2 mg L^{-1} Kin without IBA (left) and control (right); (**B**) callus induced on explants grown in culture medium containing 0.5 mg L^{-1} Kin with 0.5 mg L^{-1} IBA (left) and control (right); (**C**) plantlets produced on medium enriched with 2 mg L^{-1} Kin with 1 mg L^{-1} IBA (left) and control (right); (**D**) plantlets grown on medium enriched with 1 mg L^{-1} Kin with 1 mg L^{-1} IBA (left) and control (right); (**E**) shoot induced from callus in medium supplemented with 1 mg L^{-1} Kin with 0.5 mg L^{-1} IBA; (**F**) a plantlet produced on culture medium containing 1 mg L^{-1} IBA without Kin

(Tab. 4). Physical dehydration was more appropriate compared to osmotic dehydration pre-treatments using 0.75 M sucrose under a laminar airflow cabinet (Tab. 4, Figs 6 and 7).

DISCUSSION

In vitro propagation

Successful micropropagation through shoot multiplication, shoot elongation, and rooting on the propagated shoots is a practical approach to producing true-to-type the yew mother rootstock in large numbers for the development and conservation of this threatened medicinal-ornamental plant [Kochkin et al. 2023]. The production of a true-to-type plant in a short period is the first step in successfully studying *in vitro* propagation. The correct selection of the explant effectively obtains promising results, the composition of the culture medium, especially the type and concentration of PGRs, and the selective method of micropropagation

Fig. 3. The growth of pre-treated *T. baccata* explants in growth chamber after cold preservation and cryopreservation

(direct or indirect organogenesis and embryogenesis). The current study presents a multiplication approach for yew using cytokinin and a combination of auxin and cytokinin. Some plants have reported similar results [Aroonpong and Chang 2015, Kaviani and Negahdar 2017, Adibi Baladeh and Kaviani 2021, Li et al. 2021].

The results revealed Kin is an effective cytokinin for shoot induction and growth. The successful use of cytokinins such as 6-benzylaminopurine (BAP), zeatin (Zt), Kin, thidiazuron (TDZ), and N6-(Δ^2-isopentenyl) adenine (2-iP) has been reported for shoot multiplication and growth in yew [Ewald 2007, Abbasin et al. 2010]. Cytokinins are a class of PGRs that regulate cell division, differentiation, and shoot development. Kin specifically stimulates cell division and differentiation in plant tissues, leading to the formation and growth of shoots [Mukherjee et al. 2022]. The highest shoot multiplication in most plants, especially trees and shrubs, has been obtained on media containing low concentrations of cytokinins [Alvarez et al. 2009, Abbasin et al. 2010, Kaviani and Negahdar 2017, Adibi Baladeh and Kaviani 2021].

The optimal concentration of exogenous PGRs for maximal shoot multiplication varies between species. The main reason for this difference is each species' amount of endogenous PGRs. In the present study, the combination of Kin and IBA improved the shoot multiplication. The combination of a cytokinin and an auxin has been reported for the successful micropropagation of some ornamental trees and shrubs [Kaviani and Negahdar 2017, Sharma 2017, Dinesh et al. 2019, Li et al. 2021, Adibi Baladeh and Kaviani 2021]. Similar to the findings of the present study, in the media containing cytokinins alone or in combination with auxins, apical and axial buds produced an acceptable number of apical shoots [Aroonpong and Chang 2015, Kaviani and Negahdar 2017, Sharma 2017, Sharma et al. 2017, Dinesh et al. 2019]. In a few woody species, cytokinins, especially BAP and Kin, were effective for shoot multiplication [Sharma 2017]. The highest multiplication coefficient in *Pyracantha angustifolia*

(Franch.) C.K. Schneid., a tree species, was obtained in MS medium supplemented with 2.5 mg L^{-1} BAP [Kaviani et al. 2022]. High concentrations of cytokinins in some woody plants, including *B. hyrcana*, inhibited shoot proliferation [Kaviani and Negahdar 2017]. The positive effect of other cytokinins such as BA, Zt, and TDZ on shoot multiplication has been reported in several tree and shrub plants [Yıldırım 2012, Kereša et al. 2012, Aroonpong and Chang 2015, Fan et al. 2017, Sharma et al. 2017, Li et al. 2021].

BA is the most widely used cytokinin, and the combination of BA with NAA is the most widely used cytokinin and auxin combination for shoot induction *in vitro* [Kaviani 2015]. In *Mespilus germanica*, the simultaneous application of BA and NAA increased the number of leaves and nodes [Adibi Baladeh and

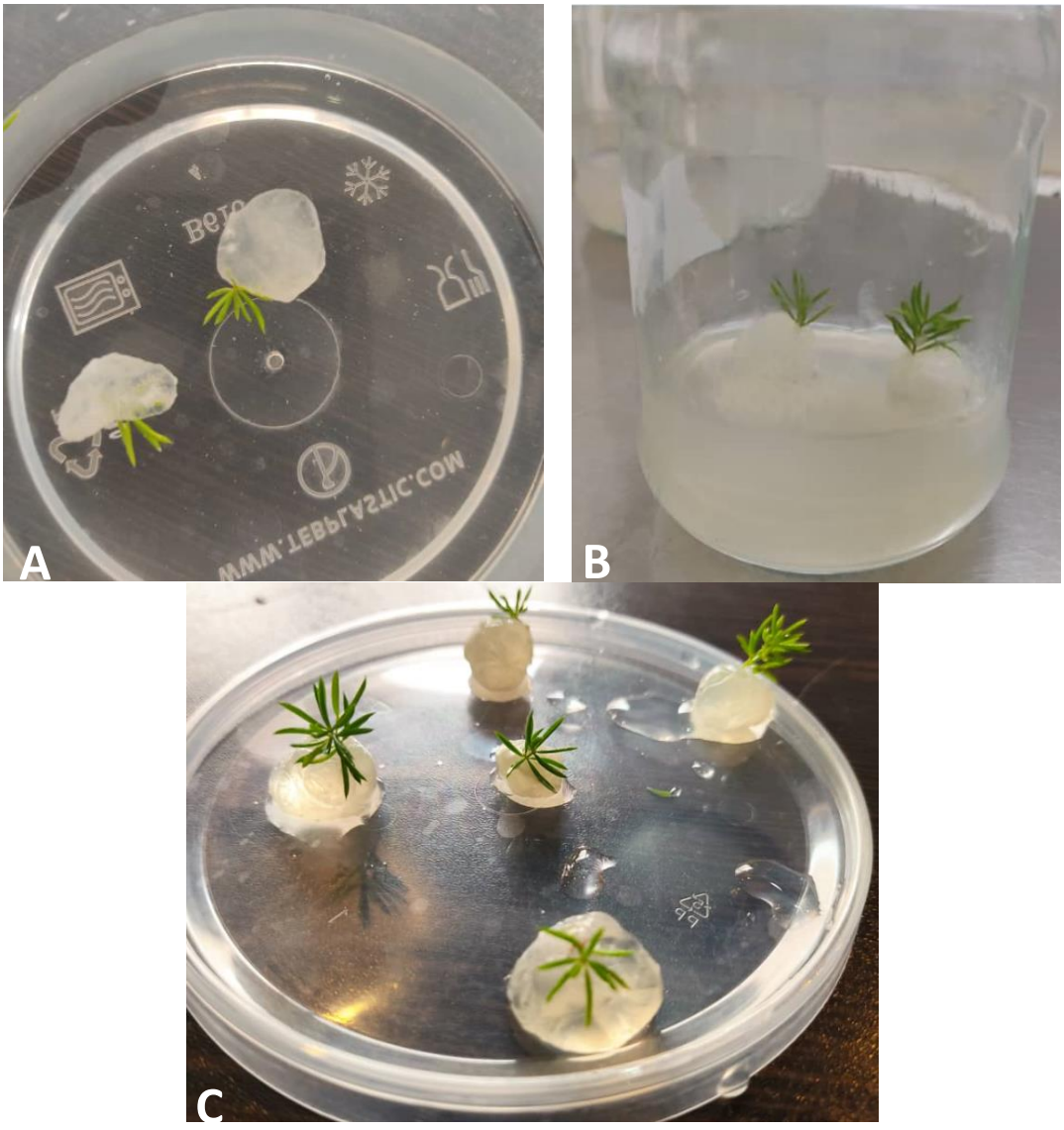

Fig. 4. (**A**) Germination of encapsulated *T. baccata* explants, (**B**) germination of encapsulated *T. baccata* explants in T_8 treatment after cold preservation, and (**C**) germination of encapsulated *T. baccata* explants pre-treated with T_6 treatment after cold preservation

Table 3. The effect of different pre-treatments on germination percentage of *T. baccata* apical buds preserved in cold conditions

Treatments	Apical buds germination (%)
T_1	$71.33^{d} \pm 10.63$
T_2	$93.66^{a\text{-}c} \pm 10.90$
T_3	$84.33^{bc} \pm 5.81$
T_4	$98.66^{ab} \pm 7.5$
T_5	$95.33^{a\text{-}c} \pm 5.426$
T_6	$88.66^{bc} \pm 3.761$
T_7	$91.33^{a\text{-}c} \pm 4.58$
T_8	$100^{a} \pm 0.00$

Values are means ±standard deviations. Different letters in the column indicate significant differences between means at ($p < 0.01$) based on the LSD test.

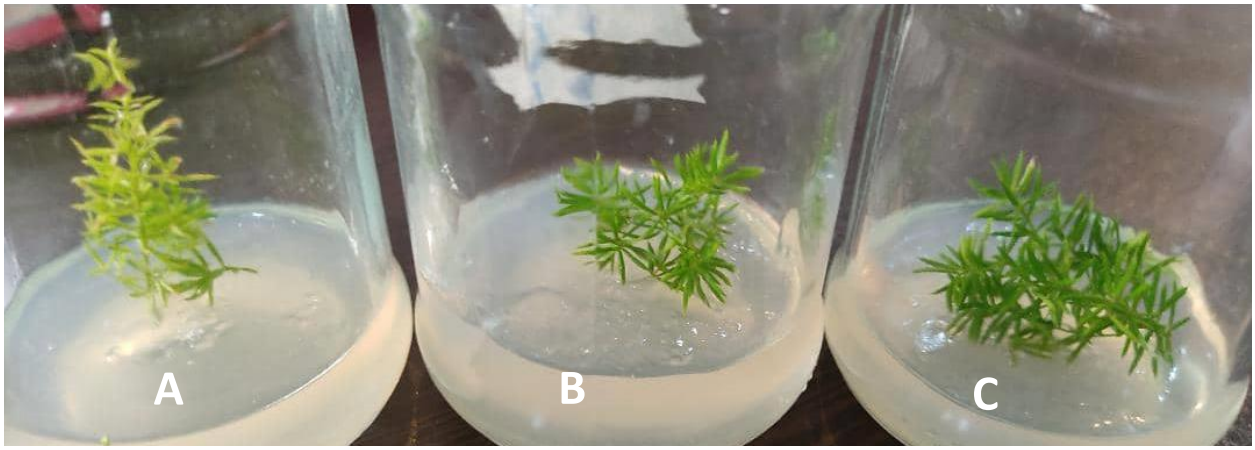

Fig. 5. The growth of germinated apical buds of *T. baccata* preserved in cold conditions. (**A**) Pre-treated with T_6 treatment, (**B**) pre-treated with T_7 treatment, and (**C**) pre-treated with T_8 treatment

Kaviani 2021]. The highest number of shoots was regenerated in *Couroupita guianensis*, a tree species, on a culture medium containing 1 mg L^{-1} of BAP and Kin with 0.5 mg L^{-1} NAA [Mahipal et al. 2016]. Generally, the optimal type and concentration of PGRs to induce the maximum number of shoots depends on the types of species, the type of explant, and the amount of endogenous PGRs.

In the present study, the positive effect of Kin and IBA in combination was shown on root induction and growth. Similar to the findings of this research, some researchers showed successful rooting on shoots using a combination of auxin and cytokinin [Kaviani and Negahdar 2017, Adibi Baladeh and Kaviani 2021]. In *B. hyrcana*, the longest root was produced in the medium containing 1 mg L^{-1} BAP and 1 mg L^{-1} NAA. Explants grown on MS medium enriched with 0.50 mg L^{-1} BAP and 1 mg L^{-1} NAA produced the highest number of roots [Kaviani and Negahdar 2017]. Auxins are the most effective PGRs for rooting cuttings and explants of tree and shrub plants in *ex vitro* and *in vitro* conditions. Auxin concentration and type significantly affect rooting percentage, root number, and root length. A literature review demonstrated that IBA is the most widely used auxin for rooting many woody species [Kaviani 2015]. In many species, the

highest rooting percentage was observed using IBA [Yıldırım 2012, Kereša et al. 2012, Sharma and Vashistha 2015, Mahipal et al. 2016, Fan et al. 2017, Dinesh et al. 2019]. In English yew, the highest percentage of root production was obtained in the medium containing 8 mg L^{-1} IBA [Abassin et al. 2010]. The highest number of roots (3.5 per micro-shoot) in *P. angustifolia* (Franch.) C.K. Schneid. was produced in the MS medium enriched with 1.5 mg L^{-1} IBA [Kaviani et al. 2022]. Better effects of IBA compared to NAA and IAA on root growth of English yew [Ewald 2007], *T. brevifolia* Nutt. [Mitchell 1997] and *T. mairei* [Chang et al. 2001] were reported. Auxins IBA and NAA stimulated rooting in many tree and shrub species [Kaviani 2015]. In *M. germanica*, 1 mg L^{-1} NAA alone or in combination with 0.50 mg L^{-1} BA effectively induced rooting [Adibi Baladeh and Kaviani 2021]. Both auxin and cytokinin were better for rooting than only one auxin in *Citrus jambhiri* Lush. [Savita et al. 2010], *B. hyrcana* [Kaviani and Negahdar 2017], and a few other woody plants [Sharma 2017]. The type, concentration, and composition of auxins required for maximum rooting are generally different in different species.

Cold preservation and cryopreservation

In the present study, the positive role of the encapsulation-dehydration technique was shown as a protective pre-treatment of germplasm (apical bud) against cold stress. So, the highest percentage of survival of germplasms after storage in the refrigerator was obtained in pre-treated apical buds by dehydration from the encapsulated explants with 0.75 M sucrose for two hours, then dehydration under the flow of clean air in a laminar airflow cabinet for two hours. This result shows the more important role of the total pre-treatments in improving germplasm survival and germination percentage after storage in cold conditions and during cultivation in the culture medium. Similar results have been reported on several ornamental and fruit species [Kaviani 2011, Kulus and Zalewska 2014, Kaviani and Kulus 2022]. Encapsulated shoot and protocorm of *Vanilla*, an endangered and rare ornamental plant, were stored for over a year without cultivation with 80% germination capacity in a slow-growth culture medium [Divakaran et al. 2006]. These samples were kept for more than seven years with annual subculture. After this period, no genetic change was observed in the preserved plants. Storage of orchid seeds at 4°C in $CaCl_2$ as a desiccant provided a suitable long-term storage (10 to 20 years). Benelli et al. [2012] reported successfully preserving other important and rare ornamental species in slow-growth conditions for 6–9 months. The normal development of the stored samples depends on the storage conditions, which affect the plant's morphology.

Encapsulating the explants allows them to withstand very harsh treatments, including dehydration. These treatments are harmful and even fatal for non-encapsulated explants. The presence of alginate beads reduces the dehydration step's speed and

Table 4. Mean comparison of the effect of different pre-treatments on germination percentage of *T. baccata* apical buds preserved in ultra-cold conditions (cryopreservation)

Treatments	Apical buds germination (%)
T_1	$59.33^{f} \pm 5.38$
T_2	$68.66^{e} \pm 6.67$
T_3	$79.66^{d} \pm 7.47$
T_4	$82.99^{cd} \pm 4.32$
T_5	$90.33^{bc} \pm 5.88$
T_6	$91.66^{ab} \pm 6.29$
T_7	$100^{a} \pm 0.00$
T_8	$87.66^{b-d} \pm 4.85$

Values are means ±standard deviations. Different letters in the same column indicate significant differences between means at ($p < 0.01$) based on the LSD test.

allows subsequent dehydration steps to reduce the moisture content of the cells before immersion in LN [Ozden-Tokatli et al. 2008, Kaviani and Kulus 2022]. In addition, the alginate matrix increases the physical support of the samples against mechanical and oxidative stress during storage and makes it easier to carry out pre- and post-storage steps in LN [Kaviani and Kulus 2022]. The present study showed the positive role of encapsulation-dehydration as a protective pre-treatment against freezing stress. In this study, the highest survival percentage of germplasms after storage in LN was obtained in pre-treated apical buds by dehydration from the encapsulated buds under the clean airflow of the laminar airflow hood for two hours. This result shows the more important role of physical dehydration and encapsulation in improving the percentage of survival and germination of germplasms after storage in LN and during cultivation in a culture medium.

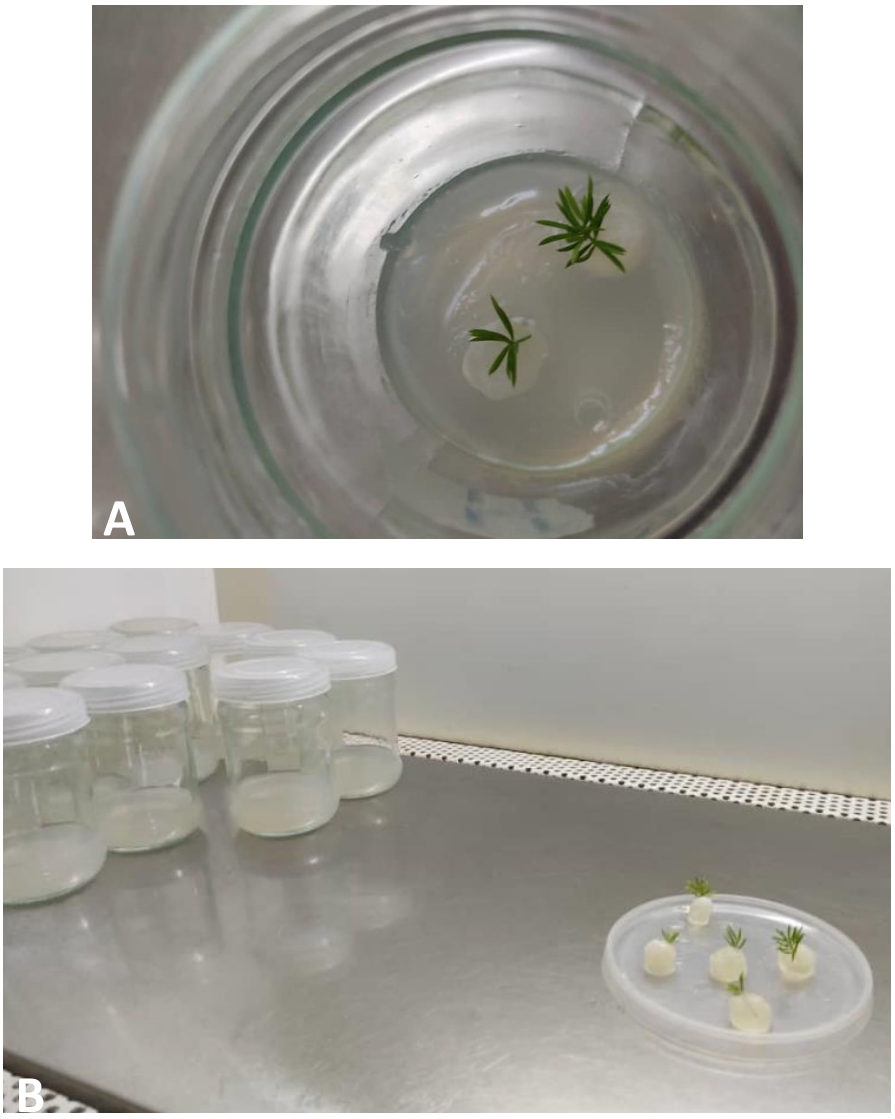

Fig. 6. (**A**) Germination of encapsulated *T. baccata* explants pre-treated with T_7 treatment after cryopreservation, and (**B**) germination of encapsulated *T. baccata* explants pre-treated with T_8 treatment after cryopreservation for subculture

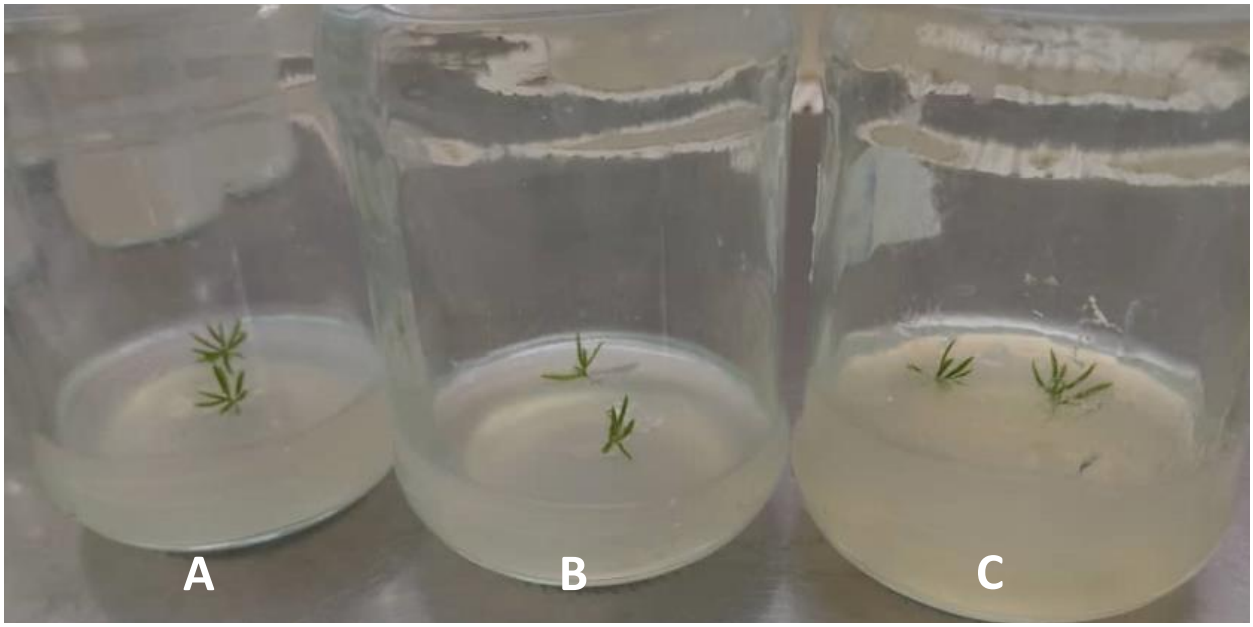

Fig. 7. Germinated apical buds of *T. baccata* preserved in ultra-cold conditions. (**A**) Pre-treated with T_6 treatment, (**B**) pre-treated with T_7 treatment, and (**C**) pre-treated with T_8 treatment

The non-pretreated apical buds had less survival after storage in LN. Similar results have been reported on several ornamental and fruit species [Kaviani 2011, Kulus and Zalewska 2014, Kaviani and Kulus 2022]. Some reports on ornamental plants, including endangered plants such as numerous cultivars of orchids, *B. hyrcana*, *F. imperialis,* and *L. ledebourii,* showed that non-encapsulated germplasm did not survive or had very little survival after cryopreservation [Kaviani 2011, Kulus and Zalewska 2014, Kaviani and Negahdar 2017, Seydi et al. 2019, Kaviani and Kulus 2022].

Germplasm preservation of *L. ledebourii* was done in very low-temperature conditions by encapsulation method and using sucrose and physical dehydration. This study strongly suggested that the survival in the embryo axis of the control was equal to zero, and that of the pre-treated with 0.75 M sucrose and one hour of dehydration under a laminar airflow hood was equal to 75%. Furthermore, it was shown that after storage in LN, survival in control seeds was zero, in seeds treated with 0.6 M sucrose and dehydrating for one hour in laminar flow was 22%, and in encapsulated seeds treated with 0.6 M sucrose and dehydrating for one hour in laminar flow was equal to 51% [Kaviani 2010]. The presence of sucrose in the capsule, in addition to dehydrating, can stimulate faster regeneration of explants after thawing [Kaviani and Kulus 2022]. Some reports indicated that encapsulation-dehydration provided better protection of explants against cold and freezing stress than other techniques [Kaviani 2011, Kaviani and Kulus 2022]. The encapsulation-dehydration technique has been widely used for ornamental plants [Kaviani and Kulus 2022]. The current study suggests using encapsulation-dehydration treatment for cold preservation and cryopreservation of yew.

CONCLUSIONS

English yew populations in their natural habitats are declining due to illegal exploitation and climate change. *In vitro* propagation of most trees and shrubs is difficult due to some problems. This research indicated that yew was propagated *in vitro* using apical buds as an explant, Kin and IBA as PGRs, and direct organogenesis as the method used. The highest shoot multiplication and root induction were obtained in media enriched with 1 mg L^{-1} IBA and 1 and 2 mg L^{-1} Kin, respectively. *In vitro* propagation is an effective and promising method to preserve

valuable plant species at risk of extinction or threat. Due to the support of numerous ornamental species, especially the valuable species in the process of extinction and the increase of the available genetic diversity, it is necessary to use modern biotechnology tools. An adequately supported explant can be stored in LN indefinitely. Combining plant tissue culture techniques and storage in cold and ultra-cold conditions can create an extensive plant gene bank in a limited space. Supporting germplasms before storage in LN plays a vital role in regenerating these germplasms after storage in LN. In the present research, it was found that the technique of encapsulation-dehydration as a pre-treatment had an effective role in the survival and germination potential of yew explants (apical buds).

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