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In Vitro Propagation of the Endangered *Kosteletzkya pentacarpos* (L.) Ledeb: Conservation Applications and Horticultural Prospects

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Abstract

Employing rare or threatened species in ornamental horticulture offers a dual benefit by promoting climate adaptation and enhancing species conservation. *Kosteletzkya pentacarpos*, an endangered halophytic species, holds potential for introduction into the nursery industry, but efficient propagation methods are lacking. The present study investigated the in vitro propagation of the species using nodal explants excised from aseptic seedlings. A two-stage in vitro culture system was tested with thidiazuron (TDZ) promoting shoot initiation at low concentrations, while higher levels induced callus formation. Transferring micro-shoots to hormone free-, Murashige and Skoog medium (MS) promoted the highest shoot multiplication and elongation. The effect of sodium chloride (NaCl) on in vitro culture was also assessed, with MS media containing up to 5.0 g L^{-1} NaCl supporting successful culture establishment. Spontaneous rooting was observed during various stages of the culture process. Micro-shoots were rooted at 100.0% on half strength MS medium with or without indole-3-butyric acid, and all plantlets were successfully acclimatized in a peat–perlite substrate (1/1, *v*/*v*). Thus, the present protocol provides an efficient system for the large-scale propagation of *K. pentacarpos* serving as a valuable tool for its conservation and the potential use in the nursery industry.

Keywords: aseptic seedling; halophyte; seashore mallow; spontaneous rooting; thidiazuron

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1. Introduction

Ornamental plants, both woody and herbaceous, play an important role in providing ecosystem services, particularly in urban areas under climate change [1–12]. Conversely, the impact of climate change in floriculture and landscape gardening is increasingly investigated [13–17] in order to identify resilient varieties and innovative mitigation strategies. Using rare or threatened species for ornamental purposes could support both climate adaptation and species conservation [18,19]. A key example to that regard is the EU LIFE project Seedforce (LIFE20 NAT/IT/001468), which aims to improve the conservation status of 29 threatened species listed in Annex II of the EU Habitats Directive, currently assessed as 'unfavourable-inadequate' (U1; 19 taxa) or 'unfavourable-bad' (U2; 10 taxa) [20]. The

project integrates both ex situ and in situ approaches, including habitat restoration in the 76 Natura 2000 sites (SCI/SACs) selected (450,250 ha in total), germplasm collection and propagation of at least 50,000 individuals for the reinforcement of 139 populations of the 29 target species. Additional actions involve long-term monitoring, guidelines for site and species management in collaboration with ISPRA, and agreements with site authorities to ensure population persistence. Horticultural techniques are central to these efforts, while the cultivation and commercialization of species with high ornamental value may further enhance both conservation and the floriculture sector.

Kosteletzkya pentacarpos (L.) Ledeb. (syn. *Kosteletzkya virginica* (L.) C. Presl; f. Malvaceae), commonly known as "seashore mallow" or "saltmarsh mallow", is a perennial wetland halophyte growing to 0.8–2.0 m in height. It bears elongated leaves (6.0–14.0 cm) and numerous pink flowers (5.0–8.0 cm) from July to September (Figure 1), features that contribute to its ornamental value [21–23]. Native populations of the species can be found along the Atlantic and Gulf coasts of eastern North America, the Mediterranean coasts of south Europe, and the shores of the Black and Caspian Seas [24–28]. Blanchard [29] reduced *K. virginica* (referred to North American populations) to synonymy under *K. pentacarpos* based on morphological indistinguishability between the North American and the Eurasian populations.

Figure 1. *Kostelekzya pentacarpos* in its natural habitat, and plant blooming in Punta Sabbioni (Venice), Italy, at the peak of the flowering season. Scale bar = 1 cm.

The species typically inhabits irregular flooded coastal areas and river banks, where recurring water saturation and low salinity are essential attributes of its niche. It occurs at the margins of coastal lagoons and other water bodies close to the sea, on humid, subsaline sandy or loamy soils, which may be acidic, neutral or basic, and are occasionally flooded. *K. pentacarpos* is commonly associated with reeds and rushes in coastal, particularly *Phragmites australis* (Cav.) Trin. ex Steud. and *Juncus maritimus* Lam. [30]. According to the EU Habitats Directive (92/43/EEC), plant communities hosting *K. pentacarpos* correspond to habitat 1410 "Mediterranean salt meadows (*Juncetalia maritimi*)".

As a salt-tolerant species, *K. pentacarpos* plays an important ecological role in soil stabilization and biodiversity support within coastal salt marshes. Its high protein and oil content also make it valuable for biomass production, animal feed, and phytoremediation of saline soils [26,31–33]. Since 1993, it has been cultivated in China for ecological restoration and as a potential biodiesel source on saline-degraded lands [31,32].

K. pentacarpos is threatened by agricultural expansion, urban development through land reclamation and wetland drainage, habitat loss, invasive species and climate change. It is intolerant of and restricted mainly to open areas. Studies on Spanish populations identified several factors affecting reproductive success and population dynamics. These

included a transient, shallow seedbank and the rapid maturity and longevity of the adults. Contrastingly, it was found that mature individuals are subject to a strong decrease of fecundity with age, leading to fluctuations in the production of fertile seeds [21,34]. Populations in Europe, particularly in Italy and Spain, are under pressure [21,28,29,35–40]. In Italy, the species is listed as CR A2ac (EW in Lazio, Apulia, Campania and Tuscany) [40] and it is assessed as VU (B2abi-v) in the IUCN European Union Red Lists [41]. It is included in Annex II of the EU Habitats Directive (92/43/EEC) and Annex I of the Bern Convention [39,40]. *K. pentacarpos* is also a target of the EU LIFE project Seedforce (LIFE20 NAT/IT/001468), which focuses on its relict Italian populations, currently confined to the upper Adriatic coasts (Veneto and Emilia Romagna). These populations fluctuate widely, from a few hundred to 4000 ramets [38].

For the ex-situ conservation of wild plants originating from native coastal habitats affected by salt, it is important to study the effect of sodium chloride (NaCl) in the culture medium, because its presence can be a critical factor for the establishment of an in vitro propagation protocol [42,43]. Tissue culture offers significant potential as a tool for rapid plant propagation and provides an effective alternative approach for ex situ conservation [44,45]. Furthermore, tissue culture is considered a reliable, rapid, and cost-effective method for selecting salt-tolerant plants [46]. NaCl proved critical for the successful in vitro propagation of *Salicornia europaea* L. [47] and *Salicornia brachiata* Roxb. [48] while in *Crithmum maritimum* L., higher NaCl concentrations reduced shoot proliferation but increased shoot height [49]. Similar studies revealed relatively high NaCl tolerance in rare and endangered Baltic Sea species such as *Glaux maritima* L., *Dianthus arenarius* ssp. *arenarius*, and *Linaria loeselii* Schweigg. [42]. Notably, explant responses may vary across propagation stages, as shown in *Limoniastrum monopetalum* (L.) Boiss. [46]. Available information about effective micropropagation protocols for *K. pentacarpos* remains limited. Piovan et al. [50] developed such a protocol from meristems and callus organogenesis. Shoot proliferation was significantly affected by the type of explant, the hormones and their interaction, with only leaf-derived calli producing shoots. Previously, Ruan et al. [51] had presented a similar in vitro propagation protocol of *K. pentacarpos* (under its synonym *K. virginica*), assessing callus induction on embryo axes and the subsequent induction and rooting of callus-derived shoots. More recently, the effect of salicylic acid (SA) and its derivative acetylsalicylic acid (ASA) in plant growth and development was examined on Murashige and Skoog media (MS) [52,53].

In the present study, the in vitro propagation of *K. pentacarpos* was further investigated with the aim of establishing an effective in vitro propagation protocol to achieve multiple shoot induction. Thidiazuron (TDZ) a phenylurea compound exhibiting both auxin- and cytokinin-like activity, was employed to enhance the proliferation rate of in vitro cultures derived from nodal explants, due to its effectiveness on morphogenesis response in numerous plant species [54]. Furthermore, the role of NaCl in the culture medium was evaluated to explore its relevance for the propagation and the ex-situ conservation of this halophytic species. We hypothesize that optimizing TDZ concentrations, in combination with controlled NaCl supplementation, can significantly improve shoot proliferation. This work provides the first systematic attempt to establish an effective and easily reproducible protocol for the rapid micropropagation of *K. pentacarpos*, supporting its dual potential as an ornamental species for floriculture and as a target for conservation programs.

2. Materials and Methods

2.1. Plant Material

Seeds were collected from a wild population of *K. pentacarpos* in Punta Sabbioni (45°25′41.43″ N, 12°25′44.0364″ E, Venice, Veneto Region, Italy) in September 2020 and

2021. Fully ripened seeds were manually separated from the fruits after their maturation on the mother plants, indicating their complete ripeness [55]. The seeds were stored in airtight-sealed aluminium bags at the seed bank of the Botanical Garden of Padova, maintained at −18 °C and 7.0% relative humidity. Seeds collected in 2020 and 2021 were stored for 18 and 6 months respectively, prior to the germination trials. Before disinfection, the pericarp was removed manually, and seeds were surface sterilized by immersion in 20% (*v*/*v*) commercial bleach solution (4.6% *w*/*v* sodium hypochlorite; NaOCl) for 10 min, followed by three rinses of 3 min each in sterile distilled water. They were then sown in 9-cm plastic Petri dishes containing hormone-free (Hf), half-strength MS medium. Actively growing seedlings produced from in vitro germinated seeds at 15, 20 and 25 °C [56] were used as the source for contamination-free stock planting material and the establishment of initial cultures of *K. pentacarpos* used for the in vitro propagation studies.

2.2. Effect of TDZ and 1-Naphthaleneacetic Acid (NAA)

The initial establishment of the in vitro cultures was based on data from previous studies [53]. One-node explants (0.6 cm in length) were excised from 40-day-old aseptic seedlings and cultured on MS hormone-free medium (Hf) (first subculture). A second subculture was carried out to increase the propagating material. After these two subcultures, the effect of TDZ was evaluated as follows: one-node explants were cultured on MS medium supplemented with TDZ (0-control, 0.01, 0.05, 0.1, and 0.5 mg L^{-1}; Stage A). Subsequently, explants excised from Stage A (origin medium) were transferred to MS Hf medium (Stage B). In a separate experiment, the combined effects of TDZ and NAA were evaluated. NAA was applied at concentrations of 0.1 and 0.5 mg L^{-1}, while TDZ was used at concentrations of 0, 0.01, and 0.05 mg L^{-1}, resulting in all possible combinations of these treatments. An additional treatment containing no hormones served as the control. Subculturing was performed every 40 days on MS medium, either hormone-free or supplemented with TDZ, in the absence or presence of NAA.

2.3. Effect of NaCl on In Vitro Morphogenesis

To investigate the effects of NaCl on in vitro morphogenesis, the concentration of NaCl both during the stage of micro-shoot production and during the root induction stage was studied in detail. Nodal explants derived from micro-shoots grown on Hf (40 days old), MS medium were cultured on MS supplemented with 0 (control), 1.0, 5.0, 10.0, 15.0, 20.0 or 25.0 g L^{-1} NaCl.

2.4. In Vitro Rooting and Effect of NaCl on In Vitro Rooting

Micro-shoots 2.0–2.5 cm long (40 days old) were transferred for rooting onto half-strength MS media (MS/2) containing 0.0, 0.5, 1.0, or 2.0 mg L^{-1} indole-3-butyric acid (IBA). In another experiment, micro-shoots, 2.0 to 2.5 cm long (40 days old), grown on Hf, MS were cultured for rooting on half-strength MS medium supplemented with 0.0, 1.0, 5.0, 10.0, 15.0, or 20.0 g L^{-1} NaCl.

2.5. In Vitro Culture Conditions

In vitro cultures were established in Magenta GA-7 vessels (7.2 cm × 7.2 cm × 10.0 cm, (Merk Life Science S.r.l., Milano, Italy), with five explants installed per vessel (50 mL medium each). For NaCl- media tubes with 10 mL each were used. The media were supplemented with 30.0 g L^{-1} sucrose and solidified with 8.0 g L^{-1} agar. The pH of the medium was adjusted to 5.7 to 5.8 before autoclaving at 121 °C min for 20 min. All cultures were maintained under controlled conditions in a plant growth chamber set at 25 ± 2 °C, with a 16-h photoperiod provided by cool-white, fluorescent lamps at a light intensity of 37.5 µmol m^{-2} s^{-1}.

2.6. Acclimatisation

Spontaneous rooted and IBA- rooted plantlets, measuring 2.5–3.5 cm in length, were thoroughly rinsed under running tap water to remove any residual medium and then transferred to 1500.0 mL plastic containers (eight plantlets per container, 10 containers in total). Each container was filled with a peat: perlite substrate (1:1, *v*/*v*; peat: pH 5.5–6.5, Klasmann-Deilmann GmbH, Geeste, Germany; perlite: particle size 1.0–5.0 mm, Perloflor, Isocon S.A., Athens, Greece). To maintain high humidity, the containers were covered with transparent plastic wrap and placed in a growth chamber set at 25 ± 2 °C with a 16-h photoperiod under cool-white, fluorescent lamps, providing a light intensity of 37.5 μmol m^{-2} s^{-1}. One week later, the plastic covers were removed, and the containers were moved to a bench of heated glasshouse at the Laboratory of Floriculture and Landscape Architecture, Agricultural University of Athens (37°58′58.0″ N, 23°42′19.2″ E). Following, the plants were transferred to 1500.0 mL plastic pots filled with a 1:1 (*v*/*v*) peat:perlite substrate and fertilized weekly with a 2.0 g L^{-1} solution of a complete water-soluble fertilizer (Nutrileaf 60, 20-20-20; Miller Chemical and Fertilizer Corp., Hanover, PA, USA). Four weeks after acclimatisation, the plants were transplanted in 1.1 L plastic pots containing same substrate for further growth and were fertilized weekly with 2.0 g L^{-1} Nutrileaf 20–20–20. The final step, conducted two months later, involved assessing the survival rate of the plants.

2.7. Data Collection and Statistical Analysis

All establishment, multiplication and rooting data were collected after 40 days of culture, which was the duration of all subcultures performed during the experiments. The data included the total explant response (percentage of explants that generated micro-shoots), the total number of micro-shoots, long micro-shoots (>0.5 cm; LS) and short shoots (<0.5 cm; SS), the mean length and node number of LS, the percentage of rooted explants, the length and number of roots per explant. Furthermore, the multiplication index (MI) [57] was calculated:

$$\text{MI} = [(\text{explant response} \times \text{LS number} \times \text{LS length})/(0.6 \times 100)]$$

Data of successfully established plantlets were recorded 21 days after the start of the process of rooted plantlets. The calculation of the plants' survival rate was recorded two months later.

The complete randomized design was applied throughout the study, and the significance between treatments was assessed by one-way analysis of variance (ANOVA). Percentage data were arcsine-transformed before statistical analysis was performed. The treatment means were compared using Tukey HSD at $p < 0.05$ (JMP 14.0 software, SAS Institute Inc., Cary, NC, USA, 2013). All the experiments were replicated twice with similar data and were pooled for statistical analysis. The number of replicates (explants) per treatment is indicated in the corresponding tables.

3. Results

3.1. Effect of Thidiazuron

Following the successful establishment of aseptic plant material, a two-stage in vitro culture system was employed to mitigate the inhibitory effects of TDZ on micro-shoot elongation during the multiplication phase. Initially, the effect of varying TDZ concentrations on shoot organogenesis was assessed. Upon completion of this stage, the effect of explant origin on shoot formation was estimated using Hf, MS medium (Table 1). Explant shoot formation reached 100% on media lacking TDZ or supplemented with 0.01 or 0.05 mg L^{-1} TDZ, but declined progressively with increasing TDZ concentration (Table 1; Figure 2). The control medium yielded the highest multiplication index (MI, 11.9), whereas supplementation with 0.01 mg L^{-1}

TDZ reduced the MI to 4.8, despite this treatment producing the greatest number of long shoots (LS) (Table 1). The reduction of MI was due to the significantly reduced shoot length (1.5 cm) observed in the micro-shoots compared to that of shoots formed in Hf medium (6.5 cm). In media supplemented with higher concentrations than 0.01% TDZ the shoot formation reduced to 17.0%. In the second stage, it was revealed that the presence of TDZ in the origin- medium affected shoot formation on varying morphometric data of the cultures. Explants originating from medium containing 0.01 mg L^{-1} TDZ exhibited higher numbers of long shoots (LS, 1.4), greater LS shoot length (9.0 cm), and increased node number (6.0). These values contributed to an enhanced multiplication index (MI) of 18.9, representing a 50.0% increase compared to the MI observed in hormone-free (Hf) medium (11.4) (Table 1; Figure 2).

Table 1. Effects of varying thidiazuron (TDZ) concentrations on shoot organogenesis from nodal explants of *Kosteletzkya pentacarpos* under a two-stage (stage A and B) in vitro culture system using Murashige and Skoog (MS) medium. (A) presents shoot induction and callus formation data from explants cultured on MS medium supplemented with different concentrations of TDZ or devoid of TDZ; (B) presents explant responses as affected by the composition of the initial culture medium.

Stage A; Initial Culture: Culture on MS Containing Various TDZ Concentrations							
TDZ (mg L^{-1})	Response (%)	LS † Number	SS †† Number	LS Length (cm)	Node Number	MI †††	Callus Formation (%)
-(control)	100.0	1.1 b	1.5 b	6.5 a	3.5 a	11.9 a	40.0 b
0.01	100.0	1.9 a	1.5 b	1.5 b	1.6 b	4.8 b	100.0 a
0.05	100.0	0	2.5 a	<0.5	-	-	100.0 a
0.1 ††††	17.0	-	1.0	<0.5	-	-	100.0 a
0.5 ††††	17.0	-	1.0	<0.5	-	-	100.0 a
F ONE-WAY ANOVA	-	*	*	***	***	***	***
Stage B; Effect of Origin Medium on Subculture Response: Explants Excised from Each Stage A (Initial Culture) Treatment Were Transferred to Hf, MS Media							
Origin TDZ (mg L^{-1})	Response (%)	LS number	SS number	LS length (cm)	Node number	MI	Callus Formation (%)
-(control)	100.0 a	1.1 b	1.3 b	6.2 b	3.8 b	11.4 b	30.0 b
0.01	90.0 b	1.4 a	1.1 b	9.0 a	6.0 a	18.9 a	20.0 b
0.05	30.0 c	1.2 b	7.5 a	1.6 c	1.3 c	0.8 c	100.0 a
0.1 ††††	-	-	-	-	-	-	100.0 a
0.5 ††††	-	-	-	-	-	-	100.0 a
F ONE-WAY ANOVA	***	*	***	**	***	***	***

† LS: Long shoots (>0.5 cm); †† SS: Short shoots (<0.5 cm); ††† MI = [(explant response × LS number × LS length)/(0.6 × 100)]; †††† These treatments were excluded from one-way ANOVA of response, shoot numbers/length/node, due to low or zero response; they included only in one-way ANOVA of callus formation. *, **, ***, significant at $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$, respectively; mean separation in columns by Tukey's HSD at $p \leq 0.05$; mean values followed by the same letter are not significantly different at $p \leq 0.05$; $n = 35$.

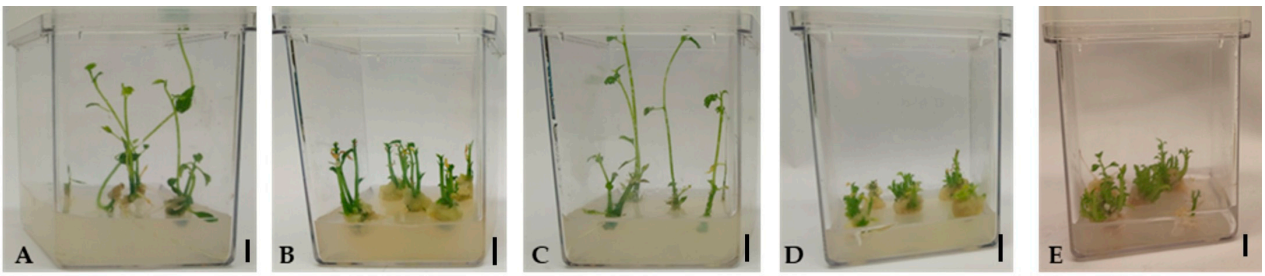

Figure 2. Shoot and callus formation of *Kosteletzkya pentacarpos* nodal explants cultured on Murashige and Skoog medium (MS): hormone-free (Hf) (**A**); supplemented with 0.01 mg L^{-1} TDZ (**B**); shoot formation on Hf, MS from explants previously cultured on MS containing 0.01 mg L^{-1} TDZ (**C**); multiple shoot and callus formation on MS supplemented with 0.1 mg L^{-1} NAA (**D**), or 0.5 mg L^{-1} NAA and 0.005 mg L^{-1} TDZ (**E**). Scale bar = 1 cm.

In a separate, one-stage experiment, the mitigation of the inhibitory effects of TDZ was assessed through the effect of varying NAA concentrations (Table 2). Explant shoot formation reached 100% on every medium tested. Again, the multiplication index (MI) was highest in the hormone-free medium (15.0) but decreased to significantly lower values in media supplemented with NAA and/or TDZ, with no perceptible influence in the number of long lateral shoots (LS) (Table 2). In contrast, the medium containing 0.05 mg L^{-1} TDZ and 0.5 mg L^{-1} NAA produced a significantly higher number (5.6) of short lateral shoots. The reduction of MI was attributed to the significantly reduced shoot length and node number compared to that of shoots formed in the hormone-free medium, with no mitigating influence by the presence of NAA at any concentration tested.

Table 2. Effects of combined 1-naphthaleneacetic acid (NAA) and thidiazuron (TDZ) concentrations, on shoot organogenesis from nodal explants of *Kosteletzkya pentacarpos* in an in vitro culture system using Murashige and Skoog (MS) medium.

NAA (mg L^{-1})	TDZ (mg L^{-1})	Response (%)	LS † Number	SS †† Number	LS Length (cm)	Node Number	MI †††	Callus Formation (%)
-	-	100.0	1.2	2.0 b	7.5 a	4.0 a	15.0 a	40.0 c
0.1	-	100.0	1.0	1.4 b	3.5 b	2.8 b	5.8 c	80.0 b
0.1	0.01	100.0	1.0	1.7 b	1.8 b	1.5 c	3.0 c	100.0 a
0.1	0.005	100.0	1.0	1.5 b	1.8 b	1.0 d	3.0 c	100.0 a
0.5	-	100.0	1.0	1.1 b	4.9 b	3.9 a	8.2 b	80.0 b
0.5	0.01	100.0	1.0	2.8 b	1.7 b	1.6 c	2.8 c	100.0 a
0.5	0.005	100.0	1.2	5.6 a	2.4 b	2.0 c	4.8 c	100.0 a
$F_{ONE-WAY\ ANOVA}$		-	ns	***	***	***	***	***

† LS: Long shoots (>0.5 cm); †† SS: Short shoots (<0.5 cm); ††† MI = [(explant response × LS number × LS length)/(0.6 × 100)]; ns, ***: non-significant or significant at $p \leq 0.001$; mean separation in columns by Tukey's HSD at $p \leq 0.05$; mean values followed by the same letter are not significantly different at $p \leq 0.05$; $n = 50$.

Both plant growth regulators (PGRs) induced prolific callus formation, most notably when combined in the medium (Table 2, Figure 2).

In the experiment that evaluated the influence of NaCl on in vitro rooting and shoot development, we noticed that NaCl can exhibit inhibitory effects that are concentration-dependent (Table 3). Explant shoot development was 100% only for the two lowest concentrations of NaCl tested (1.0 and 5.0 g L^{-1}), with high rates of non-reacting, leafless explants in the two highest concentrations (20.0 and 25.0 g L^{-1}). The number of shoots produced was not significantly different between the treatments, with almost all explants producing no lateral shoots. Both shoot length and number of nodes were negatively affected by NaCl concentrations higher than 1.0 g L^{-1}, with a median just one node of less than 0.5 cm length in media containing more than 5.0 g L^{-1} NaCl. Spontaneous rooting was seriously affected by the presence of NaCl, with no production of roots in 10.0 g L^{-1} or higher concentrations of NaCl (Table 3; Figure 3).

Table 3. Effect of NaCl at varying concentrations on the in vitro response of *Kosteletzkya pentacarpos* explants, cultured on Murashige and Skoog medium (MS).

NaCl (g L^{-1})	Response (%)	Shoot Number	Shoot Length (cm)	Node Number	Spontaneous Rooting (%)	Root Number	Root Length (cm)
0	100.0 a	1.1	3.9 a	3.2 a	65.0 a	2.3	3.6
1.0	100.0 a	1.0	2.8 a	3.2 a	60.0 a	2.1	3.0
5.0	100.0 a	1.0	1.4 b	2.5 ab	40.0 b	1.6	2.6
10.0	75.0 b	1.0	0.5 b	1.0 c	-	-	-
15.0	45.0 c	1.0	0.2 b	1.0 c	-	-	-
20.0	25.0 d	1.0	0.1 b	-	-	-	-
25.0	30.0 d	1.0	0.1 b	-	-	-	-
$F_{ONE-WAY\ ANOVA}$	***	ns	***	***	***	ns	ns

ns, ***: non-significant or significant at $p \leq 0.001$; mean separation in columns by Tukey's HSD at $p \leq 0.05$; mean values followed by the same letter are not significantly different at $p \leq 0.05$; $n = 48$.

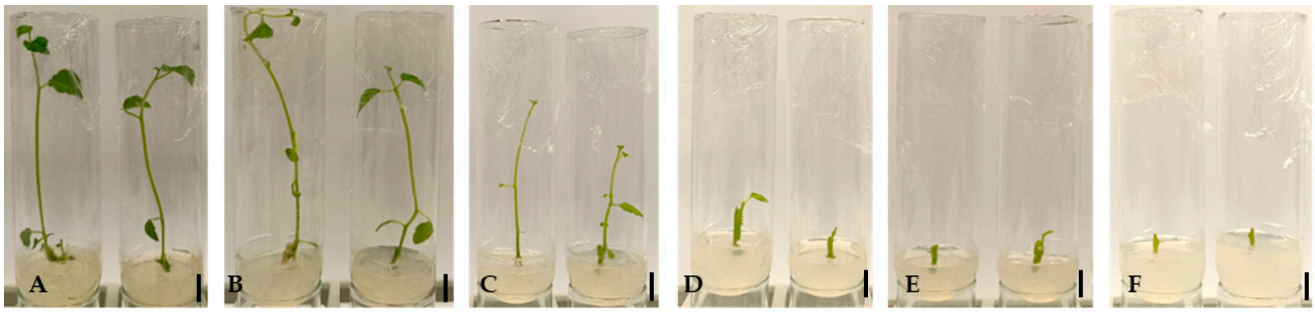

Figure 3. Shoot formation in nodal explants of *Kosteletzkya pentacarpos* cultured on Murashige and Skoog medium (MS), either hormone-free (Hf) (**A**), or supplemented with: 1.0, 5.0, 10.0, 15.0 or 20.0 mg L^{-1} NaCl (**B**–**F**). Scale bar = 1 cm.

3.2. In Vitro Rooting and Effect of NaCl on In Vitro Rooting

Spontaneous rooting was observed during the in vitro culture on Hf medium reaching to 100% (Figure 4A). In contrast, the rooting percentage on media supplemented with TDZ was 0% during Stage A. Notably the presence of TDZ in the origin medium had a significant effect on root induction during subsequent culture. At 0.01 mg L^{-1} TDZ, rooting was not suppressed, and 92.0% of shoots developed spontaneous roots with an average length of 1.9 cm during the following in vitro culture.

Figure 4. (**A**) Spontaneous root formation in *Kosteletzkya pentacarpos* micro-shoots cultured, on hormon-free, full-strength Murashige and Skoog medium;(**B**) Acclimatized plantlets after four weeks of trans-plantation into a peat: perlite (1:1, *v*/*v*) substrate; (**C**) Surviving plantlets after eight weeks in the same substrate as above; (**D**) A three-month-old acclimatized *Kosteletzkya pentacarpos* plant at the beginning of flowering in early July 2025, grown in a peat:perlite (1:1, *v*/*v*) substrate. Scale bar = 1 cm.

Micro-shoots cultured on half-strength MS medium (MS/2), either hormone-free or supplemented with IBA at 0.5, 1.0 or 2.0 mg L^{-1}, achieved 100% rooting percentage regardless of treatment (Table 4). The presence of IBA did not significantly affect root number or root length, which ranged from 1.9–2.2 roots of 1.6–2.5 cm length, respectively (Table 4). On the other hand, increasing the NaCl concentration to 5 g L^{-1}, reduced the rooting percentage: micro-shoots rooted at 100% on Hf, MS/2 medium or when supplemented with 1.0 g L^{-1} NaCl, whereas at 5.0 g L^{-1} NaCl the rooting percentage was

80.0% (Table 5). No rooting occurred at 10.0 g L^{-1} NaCl or higher concentrations. Root number and length were not affected by the presence of NaCl (Table 5).

Table 4. Effect of varying concentrations of indole-3-butyric acid (IBA) in Murashige and Skoog medium (MS), on root induction of micro-shoots of *Kosteletzkya pentacarpos*.

IBA (mg L^{-1})	Rooting Percentage (%)	Root Number	Root Length (cm)
0	100.0	1.9	2.5
0.5	100.0	2.5	1.6
1.0	100.0	1.9	1.9
2.0	100.0	2.2	1.9
$F_{ONE-WAY\ ANOVA}$	-	ns	ns

ns, non-significant; *F* values represented by ns indicate no significant differences at $p \leq 0.05$; mean separation in columns by Tukey's HSD at $p \leq 0.05$; $n = 48$.

Table 5. Effect of varying concentrations of sodium chloride (NaCl) in half-strength Murashige and Skoog medium (MS), on root development in micro-shoots of *Kosteletzkya pentacarpos*.

NaCl (g L^{-1})	Rooting Percentage (%)	Root Number	Root Length (cm)
0	100.0 a	2.5	3
1.0	100.0 a	2.4	3.2
5.0	80.0 b	1.9	2.1
10.0	-	-	-
15.0	-	-	-
20.0	-	-	-
$F_{ONE-WAY\ ANOVA}$	**	ns	ns

ns, **: non-significant or significant at $p \leq 0.01$; mean separation in columns by Tukey's HSD at $p \leq 0.05$; mean values followed by the same letter are not significantly different at $p \leq 0.05$; $n = 48$.

3.3. Acclimatisation

All rooted plantlets, regardless of the medium in which rooting was induced, survived transplantation to a peat:perlite (1:1, *v*/*v*) substrate, with 100.0% survival after three weeks (Figure 4A,B). Transplanted plants in 1.1 L pots were successfully established after two months (Figure 4C,D).

4. Discussion

TDZ has a variable effect when it is added into MS media and can result in higher multiplication rates in several species, such as *Ocimum basilicum* L. [58], *Mentha arvensis* L. [59] and *Mertensia maritima* (L.) Gray [60]. In other species, it can lead to the presence of abnormalities in shoot formation e.g., in *Ebenus sibthorpii* DC. [57], in *Thymus moroderi* Pau ex Martínez [61] and *Sideritis leucantha* Cav. subsp. *leucantha* [62]. As per the results of the first experiment shown, *K. pentacarpos* is highly sensitive to the presence of TDZ in the shoot regeneration medium, with the multiplication index of its lowest concentration tested (4.8) being less than half of the one achieved in a hormone-free medium (11.9). As noted in other sensitive species, the shoots produced have very short lengths and a deformed, chlorotic and/or vitreous appearance, with most leaves falling shortly after their appearance [57].

On the other hand, it has been reported that a two-stage in vitro culture system has been successfully used to mitigate the deleterious effects of TDZ in the micropropagation of ×*Malosorbus florentina* Zucc., a rare and endangered native tree of Greece [63], and *Sphaerophysa kotschyana* Boiss., another endangered species in Turkey [64]. Using a hormone-free MS medium for the second stage, in *K. pentacarpos* this was confirmed only for the medium containing 0.01 mg L^{-1} TDZ, with the explants derived from it exhibiting the highest multiplication index (18.9). The presence of high concentrations of TDZ have been reported to inhibit both shoot and root development in excised microshoots, likely due to a 'carry-over' effect of cytokinins from the shoot proliferation medium [65]. Despite more than four decades of TDZ application, its precise mechanism of action remains unclear, and

TDZ-induced metabolic processes are still not fully understood. Erland [66] proposed six hypotheses regarding the potential mechanisms underlying TDZ activity. The formation of inactive or storage conjugates of TDZ, which may subsequently release plant growth regulators and secondary metabolites, could be explain the high multiplication index observed during the second stage of in vitro culture in half-strength MS medium.

The addition of different rations of auxins such as NAA in cytokinin-containing multiplication media has been investigated in several ornamental and medicinal plant species, including *Anthyllis barba-jovis* L., *Dianthus cruentus* Griseb., *Mertensia maritima* and *Aglaonema simplex* Blume, with usually positive effects on shoot formation and reduction of deformed, stout and/or vitreous shoots [60,67–69]. Contrastingly, in *K. pentacarpos* no apparent ameliorative influence of NAA was observed at any of the ratios tested, with only the number of short shoots produced being significantly higher in the medium containing 0.005 mg L^{-1} TDZ and 0.5 mg L^{-1} NAA. In both substages of the multiplication stage, the presence of increasing concentrations of TDZ led to increased induction rates and size of basal callus masses. In addition, both TDZ and NAA led to the significant reduction of the length of the shoots produced, either when one or both of them were present in the multiplication medium.

The present developed protocol released that use of TDZ in two stage in vitro system could be beneficial. The initial culture on MS with 0.01 mg L^{-1} TDZ followed by the subsequent culture on Hf MS media increased the multiplication index of the cultures. These achievements improved the suggested protocol of Piovan et al. [50].

It has been reported that during the micropropagation of halophytes, the presence of a wide range of NaCl concentrations in the medium can enhance the proliferation and/or rooting rates of the explants in such species as *Salicornia europaea* [47] and *Salicornia brachiata* [48]. In other cases, such as in the Mediterranean natives *Limbarda crithmoides* (L.) Dumort and *Limoniastrum monopetalum*, only low concentrations of NaCl, equal or lower than 5.0 g L^{-1}, stimulate the production of shoots and enhance the multiplication index [43,46]. Contrastingly, in species such as *Linaria loeselii* and *Crithmum maritimum*, the presence of NaCl has an inhibitory effect even in the lowest concentrations tested, with a subsequent gradual decrease in the quantity and quality of shoots produced in the multiplication stage [42,49]. This is also the case for *K. pentacarpos*, its explants exhibiting a concentration-dependent degradation of every multiplication attribute assessed and gradually assuming a heavily stunted, chlorotic appearance before drying up and dying. Thus, it can be assumed that the species is a facultative halophyte with a low tolerance to NaCl, an observation that is further corroborated by its occurrence in low salinity coastal marches throughout its range [22,33].

Rooting stage occurs during the last period of in vitro culture and it continues until the transfer of micro-plants to ex vitro conditions. For the establishment of a successful micropropagation system, the root initiation and further growth of the rooting system is essential. A commonly employed technique to enhance root formation involves reducing the nutrient medium strength to half or even lower concentrations [70]. Additionally, the application of auxins is often crucial in many plant species to induce rooting, with IBA being the most frequently used hormone for promoting in vitro rhizogenesis [71]. In this study micro-shoots achieved a 100.0% rooting percentage on both half-strength MS medium and various IBA- containing media resulting in satisfactory root length and number. This finding was expected, as root induction occurred spontaneously at various stages of in vitro culture, in line with previous data provided by Piovan et al. [50] and Bertsouklis et al. [53]. Spontaneous rooting has also been reported in other native species, i.e., *Aconitum chasmanthum* Stapf ex Holmes [72], *Cerastium candidissimum* Correns [73] and *Sideritis raeseri* subsp. *Attica* (Heldr.) Papan. & Kokkini [74]. Spontaneous rooting of in vitro regenerated

micro-shoots offers a distinct advantage in regeneration protocols, by eliminating the need for a separate auxin- medium for rooting. Notably, the inhibition of spontaneous rooting by TDZ observed in this study concurs with previous findings by Wang et al. [75], who also reported such inhibitory effects in *Juncus roemerianus* Scheele and *Juncus gerardi* Loisel. Additionally, the micro-shoots were able to root in NaCl- supplemented media until the concentration of 5.0 g L^{-1} NaCl. Higher concentrations resulted to total failure of micro-shoots' rooting whereas for other Mediterranean halophytes e.g., *Limbarda crithmoides* and *Limoniastrum monopetalum* [43,46] produced rooting percentages of 50.0% at 20.0 g L^{-1} and 100.0% at 15.0 g L^{-1} respectively. Consistent with the present findings, Grigoriadou and Maloupa [49] reported that in *Crithmum maritimum*, another Mediterranean halophyte, the rooting rate of shoots tips was 50.0% at 5.8 g L^{-1} NaCl- containing media, while no rooting was observed for higher NaCl concentrations. As a complex adaptive mechanism to high NaCl levels, the salt tolerance of a plant individual may differ from that of its constituent tissues and organs [42].

The final phase of a successful micropropagation protocol consists of the acclimatisation of the regenerated plantlets, which requires minimizing water stress or tissue water loss [76]. In the present study, all plantlets transplanted to a peat: perlite substrate were successfully acclimatized, regardless of the medium in which they induced roots- and a 100.0% survival rate was recorded 8 weeks after transplantation. This rate is superior to the 70.0% survival reported by Piovan et al. [50], likely due to differences during acclimatisation. Our protocol involves a gradual reduction of humidity using small plastic containers within controlled growth chamber conditions, followed by transfer to a greenhouse. This method has consistently yielded positive results in our laboratory with other species of conservation priority, such as *Calamintha cretica* [77], *Cerastium candidissimum* and *Sideritis raeseri* subsp. *Attica* [73,74]. Furthermore, our results align with similar cases of high success reported for other members of the Malvaceae family, including *Hibiscus moscheutos* L. [78] and *Hibiscus rosa-sinensis* L. [79]. This approach could be extended to other potentially ornamental species included in the Seedforce project, such as *Adenophora liliifolia* (L.) A.DC., *Eryngium alpinum* L. or *Primula palinuri* Petagna.

Regarding the presence of NaCl in culture media, it was evident that the species exhibits low tolerance to NaCl concentrations in various in vitro stages of morphogenesis. While the presence of elevated NaCl concentration in the medium can enhance in vitro shoot response and rooting in other halophytes, *K. pentacarpos* demonstrates a high in vitro propagation potential even in the absence of NaCl. This is a promising finding for the establishment both of conservation strategies and its exploitation as a new ornamental species. Future research could explore the potential use of other biotechnological methods and their applications, such as slow in vitro growth techniques and the production of artificial seeds for short, medium or long storage. Such studies and their ensuing legacy could support the conservation of the species while simultaneously enabling its use in the floriculture industry and ornamental horticulture.

These results provide valuable support to the Seedforce project, which integrates ex situ and in situ strategies and requires large-scale production of individuals of target species. More broadly, the use of native endangered species in ornamental horticulture can enhance the latter's role beyond its mere economic value, positioning floriculture as another tool to support biodiversity conservation. Ex-situ cultivation can alleviate pressure on wild populations by supplying seeds or propagules for habitat restoration projects, thereby reducing the need for field collections and their ensuing pressures on the natural populations. Moreover, incorporating native ornamentals into plantings informed by urban contexts such as in flowerbeds or in public parks, supported by appropriate information and low-impact communication methods (i.e., QR codes), can raise public awareness of

plant diversity biodiversity loss and encourage local participation in conservation projects. In this way, native ornamentals combine aesthetic appeal with ecological value, promoting sustainable gardening through their adaptation to local conditions, reflected in their drought tolerance, pest and disease resistance, and capacity to act as efficient carbon sinks.

5. Conclusions

This study demonstrated that NaCl is not required for the development of an in vitro culture system for the endangered *K. pentacarpos.* The species responded well to the proposed protocol using nodal explants in hormone-free MS medium. Nevertheless, a two-stage system employing TDZ proved beneficial: an initial culture on MS medium with low concentration of TDZ (0.01 mg L^{-1}), followed by a subculture on Hf medium, significantly increased the multiplication index. The resulting micro-shoots exhibited compact growth and a higher number of nodes. Successful rooting and acclimatisation further support the feasibility of this approach for ex situ conservation and its potential for nursery production, particularly in specialized landscape applications. Furthermore, the salt tolerance of *K. pentacarpos* could be harnessed in its use as part of multi-use plantations in degraded, low water quality environments facing climate change, reinforcing the ecological importance of *K. pentacarpos* in mitigating the impacts of sea-level rise under global warming [80,81].

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