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RESEARCH ARTICLE

Enhancing biotechnological approaches for the *in vitro* micropropagation: Protecting endangered wild tulip species in Samarkand, Uzbekistan

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Abstract

Tulipa, a genus of bulbous geophytes, boasts a globally recognized ornamental value. Anthropogenic influences have precipitated a decline in the wild populations of *Tulipa*, including those in Uzbekistan, necessitating the development of forward-looking conservation strategies. This study focuses on advancing biotechnological strategies for *in vitro* micropropagation to conserve endangered wild *Tulipa* species in Samarkand, Uzbekistan. *Tulipa ingens* and *Tulipa fosteriana*, listed as vulnerable and near threatened respectively, on the IUCN Red List, face numerous anthropogenic threats including habitat loss and overexploitation. Our research demonstrates the effectiveness of sterilization protocols, successful micropropagation from seeds and the importance of adapting to non-sterile conditions. Through meticulous experimentation, a novel sterilization approach involving a 5 % Domestos solution coupled with brief ethanol exposure yielded the highest viability rates for plant materials. Micropropagation from seeds resulted in successful shoot and microbulb formation, providing a promising conservation strategy for these rare species. The study underscores the urgency of conservation efforts in Uzbekistan, where the extent of occurrence and area of occupancy of these species classify them as endangered. Our findings contribute valuable data for *ex-situ* conservation measures, offering hope for the preservation of red-listed *Tulipa* species in the region.

Keywords

bulb propagation; culture medium; *in-vitro*; microclonal propagation; seed propagation; *Tulipa*

Introduction

Tulips (*Tulipa* L.) are renowned worldwide for their significant ornamental and aesthetic value. However, the precise number of species within the genus remains uncertain due to taxonomic challenges, classification discrepancies, a high rate of interspecific hybridization and polymorphism (1-3). *Tulipa* has 376 scientific names associated with it, of which 85 (23 %) are accepted species, 266 (71 %) are synonymized taxa and 25 (7 %) names have yet to be assessed 8ы. Presently, the primary center of diversity for wild *Tulipa*

encompasses over 65 species (4-5), with Uzbekistan alone harboring 33 species (6). Despite extensive historical research on *Tulipa* in Uzbekistan, which has focused on morphology, phytogeography (11, 12), species response to climate change (7-8) and molecular studies (9-13), the exploration of micropropagation techniques for these species remains inadequate (14).

The urgency of conservation efforts is underscored by the sobering statistics provided by the IUCN Red List, which lists over 42100 species threatened with extinction (15). In Uzbekistan, with its rich flora comprising 4384 species of vascular plants (16), 314 species have been identified as threatened (17). The rapid population growth and economic development in Uzbekistan have led to the over-exploitation of natural resources, increased urbanization rates and the fragmentation or loss of natural habitats. Consequently, 187 species (60 %) out of the 314 red-listed taxa (7.13 %) require special protection measures or are not protected at all. Among these species are 19 *Tulipa* species listed in the Red Data Book of Uzbekistan (17), including *T. fosteriana* and *T. ingens*, facing threats such as overgrazing, conversion of land for agricultural purposes and overexploitation. These challenges necessitate the formulation of new conservation strategies, including the exploration of micropropagation techniques (18). *In vitro* tissue culture techniques have emerged as effective conservation approaches for rare and endangered species. Notable contributions to the *in vitro* micropropagation of *Tulipa* species have been made by researchers (19-24). However, the *in vitro* micropropagation of wild relatives of cultivated *Tulipa* species found in Uzbekistan has yet to be developed.

The objective of this study is multifaceted, aiming to address critical gaps in the conservation efforts concerning wild *Tulipa* species, particularly focusing on *T. ingens* and *T. fosteriana*, both ornamental and red-listed species. As highlighted by the IUCN Red List and various studies (15, 18), these species face significant threats to their existence due to factors such as overgrazing, land conversion for agriculture and overexploitation.

Given their ecological importance and aesthetic value, it is imperative to develop effective conservation strategies to mitigate these threats and ensure the survival of these species in their natural habitats. Micropropagation techniques offer promising avenues for the conservation of rare and endangered plant species (25, 26). By employing *in vitro* tissue culture methods, it becomes possible to propagate plants rapidly and efficiently, thus enhancing their numbers and genetic diversity while minimizing the need for extensive collection from the wild (27, 28). Furthermore, the development of micropropagation protocols for these species holds broader implications for biodiversity conservation and ecosystem restoration efforts in Uzbekistan and beyond. As part of broader initiatives to protect and restore threatened plant species and their habitats, the successful micropropagation of wild *Tulipa* species can contribute to the conservation of entire ecosystems and the myriad of species they support.

Materials and Methods

Study Area

Samarkand, steeped in history and cultural significance, unfolds as one of Uzbekistan's oldest and most storied regions. Its expanse, covering approximately 123.8 km^2, sprawls across the southeastern landscape of the Republic. Bordered by the provinces of Kashkadarya to the south, Bukhara to the west, Jizzakh to the east and Navoi to the northwest, Samarkand enjoys strategic adjacency to key regions. Geographically, it finds itself nestled amidst the grandeur of the Pamir-Alay mountains' western fringes, cradled by the serpentine flow of the Zarafshan River. The region is embraced by the imposing Nurata and Aktau Mountain ranges of the Turkestan, guarding its northern flank, while the southern horizon is defined by the majestic Zarafshan mountain ranges. Samarkand's climate, marked by distinct continental traits, manifests in its annual precipitation, which averages between 282 to 460 mm, sustaining its diverse ecosystems and agricultural heritage.

Plant Material

Bulbs and seeds of *Tulipa ingens* Hoog and *Tulipa fosteriana* W. *Irving* were meticulously collected from the verdant environs of Omonquton and Nurabad, both nestled within the picturesque landscapes of the Samarkand region, Uzbekistan. These locales, carefully selected for their ecological diversity and representation of the region's floral richness, served as prime hunting grounds for the coveted bulbs and seeds of the targeted *Tulipa* species. In the quest for comprehensive sampling, expeditions were meticulously planned, ensuring the inclusion of varied habitats and microclimates, thereby enriching the genetic reservoir under study. Each specimen, painstakingly sourced from its natural habitat, brought with it a wealth of botanical heritage, contributing to the richness and authenticity of the study's plant material.

Procedures

Sub-procedure 1: Stratification

The seeds of the species of tulips germinate above ground. According to literature data (7), soil germination of seeds is low, ranging from 23 to 39 %. During cold stratification, their germination rate increased to 61-96 %. In a study, seeds of the studied tulip species at a temperature of 8 °C began to germinate after 33-39 days (*T. sylvestris*, *T. sprengeri*), 56-60 days (*T. urumiensis*, *T. tarda*) and 83-92 days (*T. kaufmanniana*, *T. turkestanica*) (29). It was revealed that the germination of seeds significantly depended on the species characteristics of tulips. Throughout the evolutionary process, these tulip species have developed specific adaptations to various ecological environments, influencing the duration of their germination. For species residing in forests (*T. sylvestris*) and low mountains (*T. sprengeri, T. urumiensis, T. tarda*), the period from sowing to the beginning of germination is the shortest. In contrast, for *T. kaufmanniana* and *T. turkestanica*, adapted to the harsh conditions of the

highlands, this period is the longest (29). A single bulb from the chosen taxa was segmented into 2-4 sections to directly induce callus formation. Seeds, once purified and sterilized, were placed in a meticulously optimized culture medium. The sterilization process for plant specimens was based on methodologies delineated (30), subsequently tailored and refined throughout the course of this investigation (31) .

Bulbs of the investigated species underwent stratification as per scientific protocols established for a duration of 6 weeks and were subsequently sterilized following an optimally devised sterilization scheme (32). For taxonomy analysis, each species was identified according to the accepted species' names (12, 33).

Sub-procedure 2: Sterilization

The sterilization of the bulbs followed methodologies (30), which were adapted throughout the study (31). A single bulb from the selected taxa was segmented into 2-4 parts to initiate callus formation directly. Once purified and sterilized, seeds were incubated in an optimized culture medium. The preparation of the plant materials for disinfection encompassed several stages: 1) cleansing seeds and bulbs with a soapy solution of sodium hypochlorite (NaOCl) for 20 min at room temperature, 2) immersing seeds and bulbs in $KMnO_4$ for 15 min at room temperature and 3) rinsing under running water for 50-60 min at room temperature.

The sterilization of plant samples was executed using a comprehensive suite of chemicals: Domestos solution (5 %); Fundazol (1.20 g/L, as a fungicide); streptomycin (1 g/L, as a bactericide); ethanol 70 % and silver nitrate 0.5 %. The sequence of the process is delineated in Table 1.

To enhance the efficacy of the sterilization process, we incorporated silver nitrate with hydrogen peroxide solution and several antimicrobial substances. In total, the sterilization sequence comprised nine stages, including 5 rounds of treatment with fungicides and bactericides. The regimen for isolated samples was conducted based on the type of explant. The sterilization approach presented for the seed and bulb slices of *Tulipa* was identified as a prospective method for scaling up processes.

Sub-procedure 3: Culture medium

Optimization of the culture medium was based on Murashige and Skoog and underwent further refinement throughout the study. The composition of the culture medium is a critical factor in the morphogenesis of plant materials. Typically, the medium includes macro and micronutrients, hormones, vitamins, growth regulators and other organic compounds. The media were autoclaved at 121 °C for 5 min to dissolve the agar and then 300 mL aliquots were dispensed into plastic containers by the Duchefa Biochemie Company. The culture vessels containing the media were subsequently sterilized by autoclaving for an additional 15 min at 121 °C. In the initial experiment, explants of scale, bulbs and

Table 2. The composition of the nutrient medium for micropropagation *T. fosteriana* and *T. ingens*

Component	Quantity (mg/L)	Component	Quantity (mg/L)
NH_4NO_3	1,650	$Fe_2SO_4 \cdot 7H_2O$	27.95
KNO_3	1,880	Na_2-EDTA$\cdot 2H_2O$	5.6
$NaH_2PO_4 \cdot H_2O$	370	H_3BO_3	6.2
$CaCl_2 \cdot 2H_2O$	440	KI	0.83
$MgSO_4 \cdot 7H_2O$	370	$Na_2MoO_4 \cdot 2H_2O$	0.25
KH_2PO_4	170	Benzo aminopurine	0.5
$MnSO_4 \cdot 5H_2O$	22.3	Nicotinic acid	0.5
$CoCl_2 \cdot 6H_2O$	0.025	Mezo-inozitol	100
$ZnSO_4 \cdot 7H_2O$	8.6	Agar-Agar	6.5 - 7
$CuSO_4 \cdot 5H_2O$	0.025	Hydrolysate	2
Vitamin B1	0.1	Glycine	2
Vitamin B6	0.5	Vitamin B8	0.5
Vitamin PP	0.5		

Table 1. Sterilization sequence of the bulb and seeds of *T. fosteriana* and *T. ingens*

No	Bulb sterilization		Seed sterilization	
	Process (1000 mL)	Duration	Process (1000 mL)	Duration
1	Wash in running water	1 h	Wash with 5 % solution of *Domestos* (75 mL)	20 min
2	Wash with 5 % solution of *Domestos*	20 min 6 times		
3	Wash in distilled water	7 times	Wash with fundazol fungicide (1.20 g)	10 min
4	Wash with streptomycin bactericide (1 g)	8 min	Wash with streptomycin bactericide (1 g)	8 min 3 times
5	Wash in distilled water	3 times	Wash in 70 % solution of ethanol	40 s 3 times
6	Wash with fundazol fungicide (1.20 g)	8 min	Wash in 5 % H_2O_2	50 s 3 times
7	Wash in distilled water	3 times	Wash with 0.4 % solution of silver nitrate 10 s	3 times
8	Treatment in 70 % solution of ethanol	1.5 min		
9	Wash in distilled water	3 times		

seeds were placed on the basic medium supplemented with vitamins: B1, B6, B8 and PP (Table 2).

Utilizing this culture medium composition, we successfully obtained sterile explants of the first generation from both bulbs and seeds. The progeny was maintained in DKW (Driver and Kuniyuki Walnut) medium for three months to facilitate the propagation of shoots from nodal explants. The medium was supplemented with 4.5 µM Benzylaminopurine and 5 nM IBA (Indole-3-butyric acid) to accelerate the rooting process before their transfer to the greenhouse. The specifics of the medium composition are detailed in Table 3.

The explants (bulbs) were planted under sterile conditions at 25 °C and incubated for 3 months in optimized conditions. During the incubation period, the development of callus from the explants was visually observed in almost all plant materials (Fig. 1). The subsequent phase involved the micropropagation of selected taxa using seeds. Literature reviews indicate that low temperatures (from 0 ºC to 10 ºC) alleviate the physiological dormancy of the embryo, thereby promoting relatively rapid intraseminal growth (29). In our experiments, the seeds of *T. fosteriana* began to grow after 80-120 days at 0-10 ºC and the seeds of *T. ingens* germinated earlier (78-110 days). This can be attributed to the fact that tulip seeds exhibit a deep morphophysiological type of dormancy, which is due to the underdevelopment of the embryo and a robust physiological mechanism inhibiting germination. Micropropagation of bulbs was performed in automated growing chambers with a photoperiod of 16 h of light (intensity 4-5 k Lux) and 8 h of dark conditions. The temperature within the chambers was maintained at 26 ºC with a relative humidity of 40-60 %.

Table 3. Composition of DKW medium for propagation of first generation seedlings

Component	Quantity (mg/L)	Component	Quantity (mg/L)
NH_4NO_3	1,415	H_3BO_3	4.5
Ca $(NO_3 \cdot 4H_2O)$	1,960	Na_2MoO_4	0.40
Zn $(NO_3)_2 \cdot H_2O$	17	$FeSO_4 \cdot 7H_2O$	33.60
K_2SO_4	1,560	Na_2EDTA	45
$MgSO_4 \cdot 7H_2O$	740	Tiamin HCl	2
KH_2PO_4	265	Benzo	1
$MnSO_4 \cdot 4H_2O$	33.5	Nicotinic acid	1
$NiSO_4 \cdot 6H_2O$	0.0005	Glycine	2
$CaCl_2 \cdot 2H_2O$	145	Sucrose	30
$CuSO_4 \cdot 5H_2O$	0.25	pH	5.50

Sub-procedures 4: Adaptation

Adaptation of regenerated shoots to soil conditions is one of the most critical and labor-intensive stages of microclonal propagation, on which the success and efficiency of the process directly depend. In the rooting process and subsequent planting in soil, the basic composition of the medium can be altered by reducing the concentration of salts and carbohydrates and quantitatively controlling plant hormones (elimination of cytokinin and the addition of auxin). The optimal concentration of auxin for effective shoot growth is crucial and depends on several factors. These include the inherent predisposition of shoots to root due to the species and varietal characteristics of the parent plants, the type of auxin used and the concentration and ratio of phytohormones at the stage of shoot propagation. The efficiency of auxin in rooting significantly decreases in the presence of a high dose of cytokinin in the first and second stages of micropropagation. A high concentration of cytokinin is recommended in the cultivation process, with lower concentrations of the hormone being gradually introduced in the subsequent stages (31). The size of the micro bulb is pivotal in the transfer of regenerated plants to non-sterile conditions. It has been experimentally determined that the optimal size of micro bulbs for the adaptation process to soil conditions is approximately 0.6-0.8 cm in diameter (32). In our experiment, the size of adapting micro bulbs ranged from 1.4 cm to 2.4 cm, which is consistent with the aforementioned data. In our study, transplantation of regenerated plants into non-sterile conditions or the inclusion of micro clones in the adaptation process under *ex-vivo* conditions involved the following stages: a) fully regenerated plants were carefully removed from disposable sterile containers and b) the root system with leaves was washed in a special solution of polyhexamethylene biguanide (polyhexadrin) + phosphoric acid. This solution has disinfecting properties against fungi and microbes before planting microclones in the soil substrate. It is important to note that we used sterile peat produced in Russia for the adaptation process. That is, we did not undergo additional sterilization of peat, as this could lead to the destruction of organic substances in the peat. In some cases, for the transformation of regenerants to soil substrate, the following ratios of substrates were

Fig. 1. Development of callus and organogenesis from bulb slices of *Tulipa* species utilizing Benzylaminopurine (0.5 mg/L).

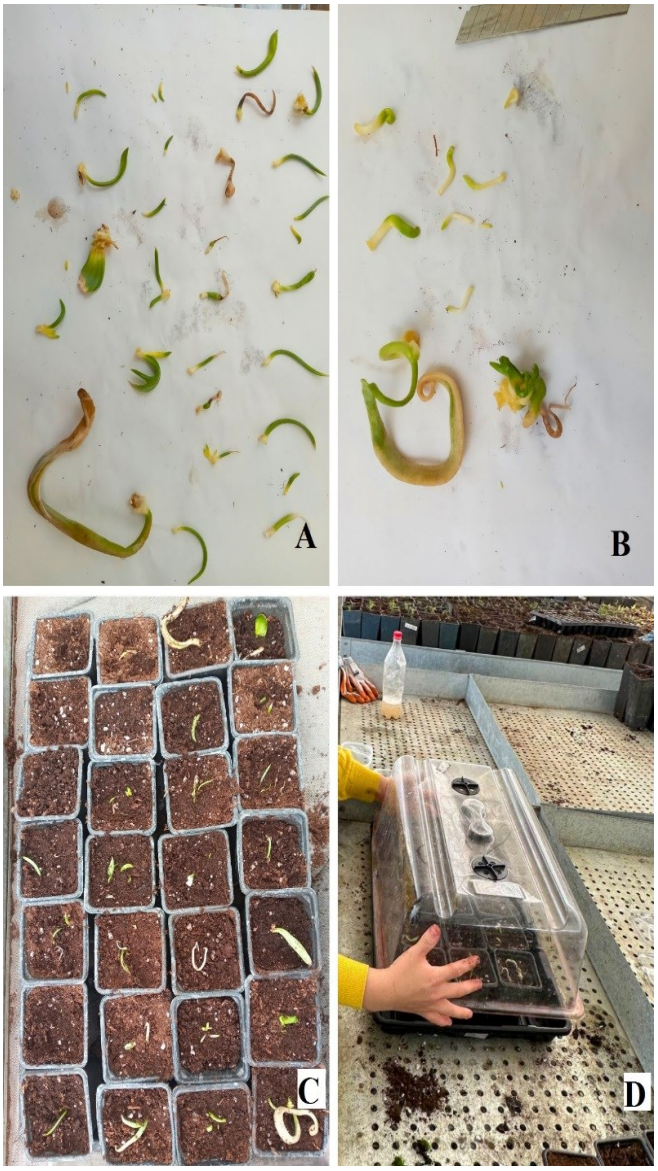

Fig. 2. Adaptation of rooting of shoots to soil conditions: A, B-dissection of explants into seedlings with bulbs, C-pots with planted tulip seedlings, D-a cassette covered with a lid with its own filtration window.

used: peat: sand (3:1); peat: turf soil: perlite (1:1:1); peat: sand: perlite (1:1:1) (30). In our study, we found that a soil consisting of 70 % peat and 30 % vermiculite was optimal for the adaptation medium for the selected species (Fig. 2).

The optimal period for planting regenerants is when the regenerant has a growing root system and developing, healthy green leaves that provide full autonomy to the plant. Notably, before the plantation of the regenerants into the selected substrate, the illumination is usually increased to 10000 Lux. Maintaining high illumination after plantation can cause chlorosis in the plants. Creating relatively high air humidity for the acclimatizing regenerated plants in the initial days is crucial for the adaptation of plants, and we recommend maintaining relative humidity around 40-60 %, which was used during the study. Significant water losses in the initial days of plantation due to the high transpiration activity of the leaves and the low absorption capacity of the roots can lead to the death of microclones. To increase the survival rate of plants in the ground, we recommend gradually increasing open-air adaptation time, such as 2-3 h per week. To prevent the rapid dehydration of leaves grown in vitro during transplantation, the application of some surface-active organic substances can be used, which substantially reduces the transpiration rate.

Results

Optimizing the stratification process for enhanced seed germination

In our inquiry, seeds embarked on a transformative journey through the stratification process, a pivotal phase vital for unlocking their innate germination potential. Set against the backdrop of controlled climatic conditions, seeds were ushered into a realm of gentle cold, with temperatures maintained at a precise 6 °C threshold. This thermal equilibrium, meticulously calibrated, served as the crucible wherein seeds were imbued with the impetus for germination, undergoing a metamorphic odyssey lasting 60 to 78 days. The seeds, embodying the genetic heritage of *T. ingens* Hoog and *T. fosteriana* W. *Irving*, responded to this orchestrated dance of temperature and time with remarkable alacrity. In a testament to the efficacy of our stratification regimen, the seeds of *T. ingens* commenced their germination after a mere 35 days, while their counterparts, the seeds of *T. fosteriana*, followed suit after 41 days. These temporal milestones, etched in the annals of botanical exploration, underscored the nuanced interplay between species-specific characteristics and environmental cues, sculpting the trajectory of germination kinetics (Table 4).

Furthermore, the bulbs of the studied species, custodians of botanical legacy and genetic resilience, were not exempted from the transformative embrace of stratification. Following time-honored protocols delineated by the venerable (34), bulbs underwent a 6 week sojourn in the realm of controlled cold, harmonizing with the primordial rhythms of nature. This temporal interlude, imbued with the essence of stratification, served as a harbinger of vitality, infusing bulbs with the latent potential for regeneration and growth. Amidst this hallowed tapestry of botanical transformation, sterilization emerged as the final act in the stratification saga. Guided by an optimally devised sterilization scheme, bulbs, having traversed the crucible of stratification, underwent a purification, cleansing them of microbial interlopers and paving the way for unencumbered growth and development.

Efficiency and efficacy of sterilization method for plant materials

In the initial phase of our research at the *in vitro* laboratory of SAG AGRO “Bog'bon”, it was imperative to identify the most optimal and effective sterilization method. This

Table 4. Germination of *Tulipa* L. seeds

Species	Period (days)		Germination (%)
	Before	Germination	
T. ingens	35	67	88
T. fosteriana	41	78	92

necessitated a comprehensive examination of existing scientific literature to gain insights into proven methodologies. Drawing upon the expertise of notable researchers (30), provided a solid foundation for our investigations (29). Their works served as valuable references, offering insights into various sterilization techniques and their respective efficacies. To systematically evaluate the efficacy of different sterilization methods, we conducted comparative experiments using a range of reagents. Among these reagents were various concentrations of Domestos solution, a household disinfectant known for its broad-spectrum antimicrobial properties. The concentrations tested included 10 %, 15 % and 20 % solutions of Domestos. Additionally, we explored a combination of ethanol (70 %), Diacid (0.1 %) and Chloramine (0.2 %) as an alternative sterilization agent. Each sterilization method was subjected to rigorous testing under controlled conditions to assess its effectiveness in eliminating contaminants from plant materials. Comparative analysis allowed us to quantitatively evaluate the performance of each sterilization agent. By systematically varying the concentration of Domestos and examining its impact on sterilization efficacy, we aimed to identify the optimal concentration for achieving the desired level of sterilization. Simultaneously, the combination of ethanol, Diacid and Chloramine was evaluated to explore alternative sterilization approaches. This combination represented a diverse spectrum of antimicrobial agents, potentially offering synergistic effects in combating microbial contamination.

Despite our initial efforts, the results obtained from the comparative experiments did not meet the stringent requirements of our scientific objectives. In response to these findings, a critical reassessment of our sterilization methods was imperative. Through meticulous analysis and further experimentation, we identified a novel approach that yielded more satisfactory outcomes.

Specifically, we observed that treating bulb slices with a 5 % concentration of Domestos for 15 min, coupled with brief exposure to ethanol (70 %) for 2 min, resulted in the highest number of viable explants while minimizing infection and necrosis. This effective optimization strategy for bulb sterilization was a significant breakthrough in our research endeavors. Conducted at the *in vitro* laboratory of SAG AGRO “Bog'bon” in Samarkand, Uzbekistan, this optimization process was meticulously designed and executed to enhance the efficiency of sterilizing agents. **Fig. 3.** Provides a comprehensive overview of the various sterilizing agents tested, along with their respective concentrations and effectiveness.

The sterilization efficiency of different concentrations of Domestos solution was compared using Kruskal-Wallis non-parametric hypothesis testing. Three concentrations of Domestos (10 %, 15 % and 20 %) were tested for their effectiveness in sterilizing plant materials. The efficiency was measured as the % of viable samples after sterilization. The Kruskal-Wallis test is used in this scenario because it is a non-parametric test suitable for comparing 3 or more independent groups when the

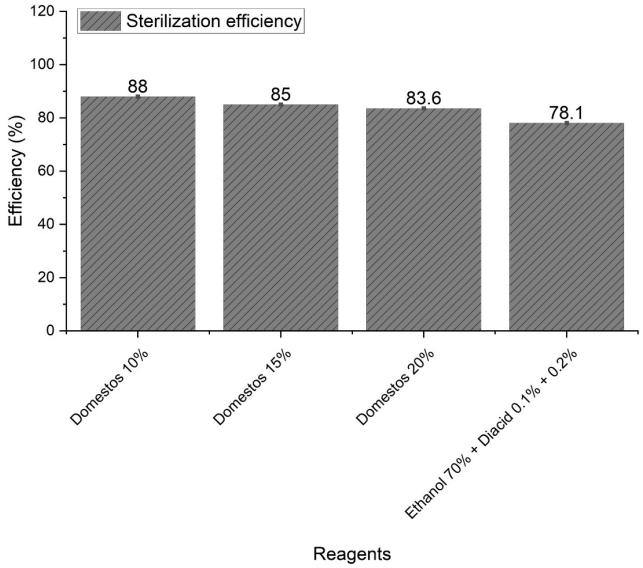

Fig. 3. Comparison of sterilization efficiency using different solutions in different concentrations.

outcome variable (efficiency in this case) is ordinal or continuous but does not meet the assumptions of normality required for parametric tests like ANOVA (34). In this study, the efficiency data for the different concentrations of Domestos were ordinal, representing the ranks of efficiency rather than precise measurements. Additionally, the sample size was relatively small, making it difficult to assess the normality assumption required for parametric tests. Therefore, the Kruskal-Wallis test is appropriate as it compares the ranks of efficiency across the different concentrations of Domestos without assuming normality. It allows for the determination of whether there are statistically significant differences in efficiency among the concentrations tested (**Table 5-7**).

The Kruskal-Wallis test was conducted to compare the sterilization efficiency across the different concentrations of Domestos. The test yielded a Kruskal-Wallis H value of 2.000 with a corresponding asymptotic significance of 0.368. The mean efficiency for each concentration was found to be 85.53 %, with a standard deviation of 2.25. The ranks of efficiency were 3.00 for 10 % concentration, 2.00 for 15 % concentration and 1.00 for 20 % concentration. Based on the Kruskal-Wallis test results, with a significance value of 0.368, which is greater than the predetermined significance level of 0.05, we fail to reject the null hypothesis (H0). Therefore, there is no statistically significant difference in the sterilization efficiency among the concentrations of Domestos tested (10 %, 15 % and 20 %). In conclusion, the analysis indicates that the sterilization efficiency does not vary significantly across different concentrations of Domestos (10 %, 15 % and 20 %). The average efficiency values for each concentration were similar, ranging from 83.6 % to 88 %. Thus, the choice of concentration within this range may not significantly affect the sterilization outcome.

Among the tested sterilizing agents, Domestos at 5 % concentration exhibited the highest efficacy, with an impressive efficiency rate of 90.8 %, leading to further exploration and application in subsequent studies. This finding underscored the importance of meticulously

Table 5. Descriptive statistics of initial reagent concentration and sterilization efficiency

Descriptive Statistics					
	N	Mean	Std. Deviation	Minimum	Maximum
Reagent concentration	3	85.5333	2.24796	83.60	88.00
Sterilization efficiency	3	2.00	1.000	1	3

Table 6. Comparison of sterilization efficiency ranks for different initial reagent concentrations

Ranks			
	Sterilization efficiency	N	Mean Rank
Reagent concentration	10 %	1	3.00
	15 %	1	2.00
	20 %	1	1.00
	Total	3	

Table 7. Test statistics for Kruskal-Wallis Test on sterilization efficiency by initial reagent concentration

Test Statistics[a,b]	
	Reagent concentration
Kruskal-Wallis H	2.000
df	2
Asymp. Sig.	.368

a. Kruskal Wallis Test

b. Grouping Variable: Sterilization efficiency

optimizing sterilization protocols to achieve desired outcomes. Additionally, the comparative analysis highlighted the superiority of the optimized approach over higher concentrations of Domestos and alternative sterilizing agents such as ethanol, diacid and chloramine. This optimized concentration of Domestos, identified through meticulous experimentation, proved to be highly efficient in sterilizing plant materials, demonstrating a notable advancement in laboratory protocols (**Fig. 4**).

The decision to utilize a lower concentration of Domestos was a strategic one, driven by the objective of achieving maximum sterilization efficacy while minimizing potential adverse effects. To assess the effectiveness of the sterilization process, 3 key criteria were selected for evaluation: the number of infected and necrotic planting samples and the number of viable explants post-sterilization. These criteria provided comprehensive insights into the overall quality and health of the sterilized plant materials.

The statistical analysis conducted on the data presented in **Tables 8, 9** and **10** aimed to determine whether there were significant differences in the effectiveness of sterilization among the tested concentrations of Domestos (5 %, 10 %, 15 % and 20 %). The results of the Kruskal-Wallis test revealed that there were no statistically significant differences in the effectiveness of sterilization across these concentrations. This implies that all tested concentrations of Domestos exhibited similar levels of effectiveness in sterilizing the plant materials. Similar to initial optimization, the statistical analysis was performed to compare the effectiveness of sterilization among different concentrations of Domestos. The null hypothesis (H0) stated that there was no difference in the level of effectiveness between the concentrations of Domestos, while the alternative hypothesis (H1) stated that there was a difference. Descriptive statistics revealed that the mean efficiency across all concentrations was 86.85 %, with a standard deviation of 3.21. The Kruskal-Wallis test was employed to assess the differences between the concentrations, yielding a test statistic (H) of 3.000 with a p-value of 0.392. Since the p-value (0.392) was greater than the significance level ($\alpha = 0.05$), the null hypothesis was not rejected. This implied that there was no significant difference in the effectiveness of sterilization among the different concentrations of Domestos (5 %, 10 %, 15 % and 20 %). Therefore, based on the statistical analysis, it was concluded that the effectiveness of sterilization with Domestos was consistent across all tested concentrations (5 %, 10 %, 15 % and 20 %), with no significant differences observed.

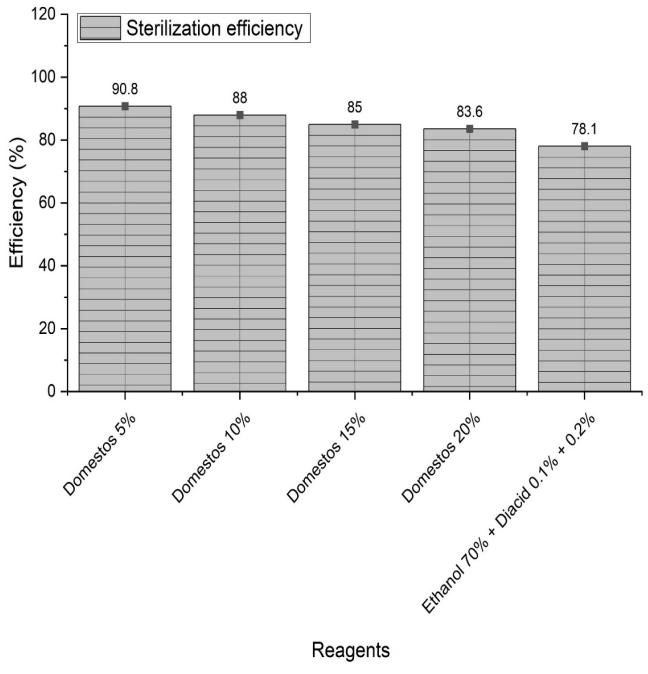

Fig. 4. Optimization sterilizing agents and their concentrations.

These findings are important as they provide valuable insights into the selection of an appropriate concentration of Domestos for sterilization protocols in the micropropagation of endangered *Tulipa* species. The lack of significant differences suggests that lower concentrations of Domestos, such as 5 % or 10 %, may be sufficient for achieving effective sterilization, thereby potentially reducing costs and minimizing potential adverse effects associated with higher concentrations. Furthermore, the consistent effectiveness of sterilization across different concentrations of Domestos enhances the

Table 8. Descriptive statistics of subsequent reagent concentration and sterilization efficiency

Descriptive Statistics					
	N	Mean	Std. Deviation	Minimum	Maximum
Reagent concentration (Domestos)	4	86.8500	3.20988	83.60	90.80
Sterilization efficiency (Domestos)	4	2.50	1.291	1	4

Table 9. Comparison of sterilization efficiency ranks for different subsequent reagent concentrations

Ranks			
	Sterilization efficiency	N	Mean Rank
Reagent concentration (Domestos)	**5 %**	1	4.00
	10 %	1	3.00
	15 %	1	2.00
	20 %	1	1.00
	Total	4	

Table 10. Test statistics for Kruskal-Wallis Test on sterilization efficiency by subsequent reagent concentration

Test Statistics[a,b]	
	Reagent concentration (Domestos)
Kruskal-Wallis H	3.000
df	3
Asymp. Sig.	.392
a. Kruskal Wallis Test	
b. Grouping Variable: Sterilization efficiency	

reliability and reproducibility of the sterilization process, contributing to the success of micropropagation efforts. This is particularly significant in conservation efforts aimed at propagating endangered plant species, where maintaining the health and viability of plant materials is paramount.

Subsequent experiments utilizing the optimized concentration of Domestos at 5 % showcased remarkable results, as highlighted in **Fig. 5**. The exposure of plant materials to Domestos at this concentration for a duration of 15 min resulted in a significant reduction in the number of infected and necrotic samples, while simultaneously increasing the number of viable explants. Specifically, the sterilization process yielded a highly sterile batch of plant materials, with an impressive viability rate of 90.8 %. Moreover, the effective optimization approach extended to bulb sterilization, where a high % of viable explants was obtained. The data presented in **Fig. 5.** Underscored the positive impact of utilizing the optimized concentration of Domestos on the viability of seeds and bulbs of *T. fosteriana* and *T. ingens*. These findings not only validated the efficacy of the optimized sterilization protocol but also reinforced the importance of systematic optimization in enhancing laboratory procedures.

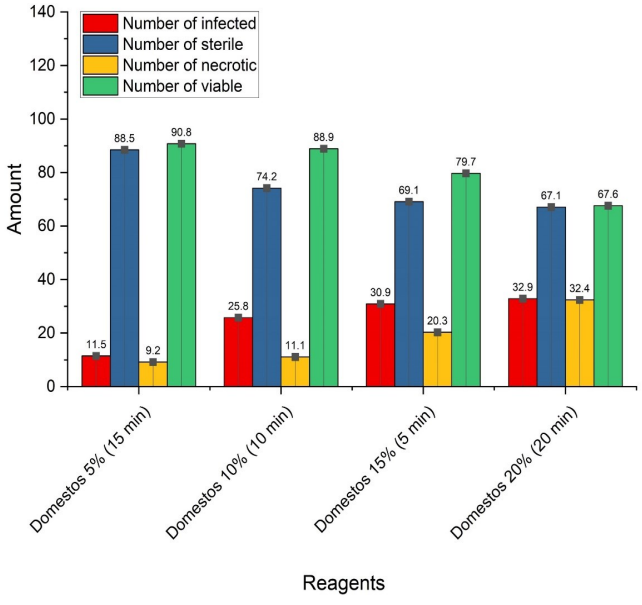

Fig. 5. The effectiveness of the effect of the concentration of the sterilizing agent on the viability of seeds and bulbs of *T. fosteriana* and *T.*

The analysis aimed to investigate the influence of 2 independent variables, Reagent concentration (X1) and Time of exposition (X2), on the dependent variable, Number of Infected (Y) (**Table 11-13**). The null hypothesis (H0) stated that both X1 and X2 do not affect Y, while the alternative hypothesis (H1) suggested that there is an influence. The significance level was set at $\alpha = 0.05$. F-Test was conducted to compare the variance explained by the regression model to the residual variance. If the F-hit value was greater than the critical F-table value, the null hypothesis would be rejected, indicating a significant influence of the independent variables on the dependent variable. The coefficient of determination (R^2) was found to be 0.950, indicating that 95 % of the variation in the Number of Infected (Y) could be explained by the independent variables Reagent concentration (X1) and Time of exposition (X2). The ANOVA results showed an F value of 9.418, with a corresponding p-value of 0.225, which was greater than $\alpha = 0.05$. Therefore, the null hypothesis was not rejected, suggesting that the independent variables collectively did not have a significant effect on the dependent variable. Furthermore, the regression coefficients for Reagent concentration (X1) and Time of exposition (X2) were 147.833 and -0.462 respectively. However, their corresponding p-values were 0.145 and 0.407, both greater than $\alpha = 0.05$, indicating that neither variable had a statistically significant effect on the Number of Infected (Y). Thus, the regression model suggests that 95 % of the variation in the Number of Infected (Y) can be explained by the independent variables Reagent concentration (X1) and Time of exposition (X2). However, the remaining 5 % of the variation may be influenced by other factors not included in the model.

Similar statistical analyses were conducted for the parameters Number of Sterile, Number of Necrotic and Number of Viable, with Reagent concentration (X1) and Time of exposition (X2) as independent variables. For the Number of sterile, the regression equation was Y = 87.433 - 147.833X1 + 0.462X2, with an R^2 of 0.950. This indicates that 95 % of the variation in the Number of sterile can be explained by the independent variables X1 and X2, while the remaining 5 % may be influenced by other factors not included in the model. Similarly, for the Number of necrotic, the regression equation was Y = -5.333 +

Table 11. Summary of regression model for predicting number of infected based on time of exposition and reagent concentration

Model Summary				
Model	**R**	**R Square**	**Adjusted R Square**	**Std. Error of the Estimate**
1	.974[a]	.950	.849	3.75588

a. Predictors: (Constant), Time of exposition, Reagent concentration (Domestos)

b. Dependent Variable: Number of infected

Table 12. ANOVA results for regression model exploring factors influencing number of infected

ANOVA						
Model		**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
1	**Regression**	265.701	2	132.850	9.418	.225[b]
	Residual	14.107	1	14.107		
	Total	279.808	3			

a. Dependent Variable: Number of infected

b. Predictors: (Constant), Time of exposition, Reagent concentration

Table 13. Co-efficients of standardized coefficients and t-values for the regression model

Coefficients						
Model		**Unstandardized Coefficients**		**Standardized Coefficients**	**t**	**Sig.**
		B	**Std. Error**	**Beta**		
1	**(Constant)**	12.567	5.737		2.190	.273
	Reagent (Domestos) concentration	147.833	34.286	.988	4.312	.145
	Time of exposition	-.462	.343	-.309	-1.347	.407

a. Dependent Variable: Number of infected

149.833X1 + 0.388X2, with an R^2 of 0.974. This suggests that 97.4 % of the variation in the Number of necrotic is accounted for by the independent variables X1 and X2, leaving 2.6 % of the variance unexplained. Likewise, for the Number of viable, the regression equation was Y = 105.333 - 149.833X1 - 0.388X2, with an R^2 of 0.974. This indicates that 97.4 % of the variation in the Number of viable can be attributed to the independent variables X1 and X2, while the remaining 2.6 % may be influenced by other factors not considered in the model. Overall, the regression analyses demonstrate a strong relationship between the independent variables Reagent concentration (X1) and Time of exposition (X2) and the number of sterile, necrotic and viable. These findings provide valuable insights into the factors influencing the sterilization process and the health of the plant materials, which is essential for optimizing micropropagation protocols and conservation efforts.

The regression equations provided insights into the specific relationships between the independent variables and the outcomes. For instance, higher reagent concentrations were associated with littler sterile fewer viable, but higher numbers of infected and necrotic plants. These findings have significant implications for optimizing sterilization protocols in micropropagation procedures, particularly for endangered plant species. By understanding how reagent concentration and exposure time affect sterilization efficiency and plant health, researchers and conservationists can develop more effective strategies for propagating and preserving rare and endangered plant species. Furthermore, the high R^2 values indicate that the regression models adequately capture the variability in the outcomes, suggesting that the selected independent variables provide meaningful explanations for the observed changes in plant health. However, it's essential to acknowledge that there may be additional factors not considered in this study that could also influence sterilization outcomes.

Fine-tuning culture medium composition for improved growth and viability

The optimization of the culture medium was a crucial aspect of the study, aimed at enhancing the growth and viability of the plant explants. After a cultivation period of 6-7 months, it was observed that 62.5 % of the explants remained viable under the *in vitro* conditions. This indicated the success of the optimized culture medium in sustaining the growth of the plant materials over an extended period. Under the *in vitro* conditions, each tulip shoot exhibited an apical meristem surrounded by the base of a single curled leaf. Following the cooling process, there was an observed intensive growth of the leaves, indicating the robustness of the culture medium in promoting plant growth and development.

The initial stages of bulb formation were characterized by the cessation of leaf growth, accompanied by the swelling of the bases of shoots and their progressive yellowing. These morphological changes served as key indicators of the bulb formation process under the optimized culture conditions (24). In the study, seed growth was observed after 116 days, with visible appearances of seed sprouts becoming evident after 131

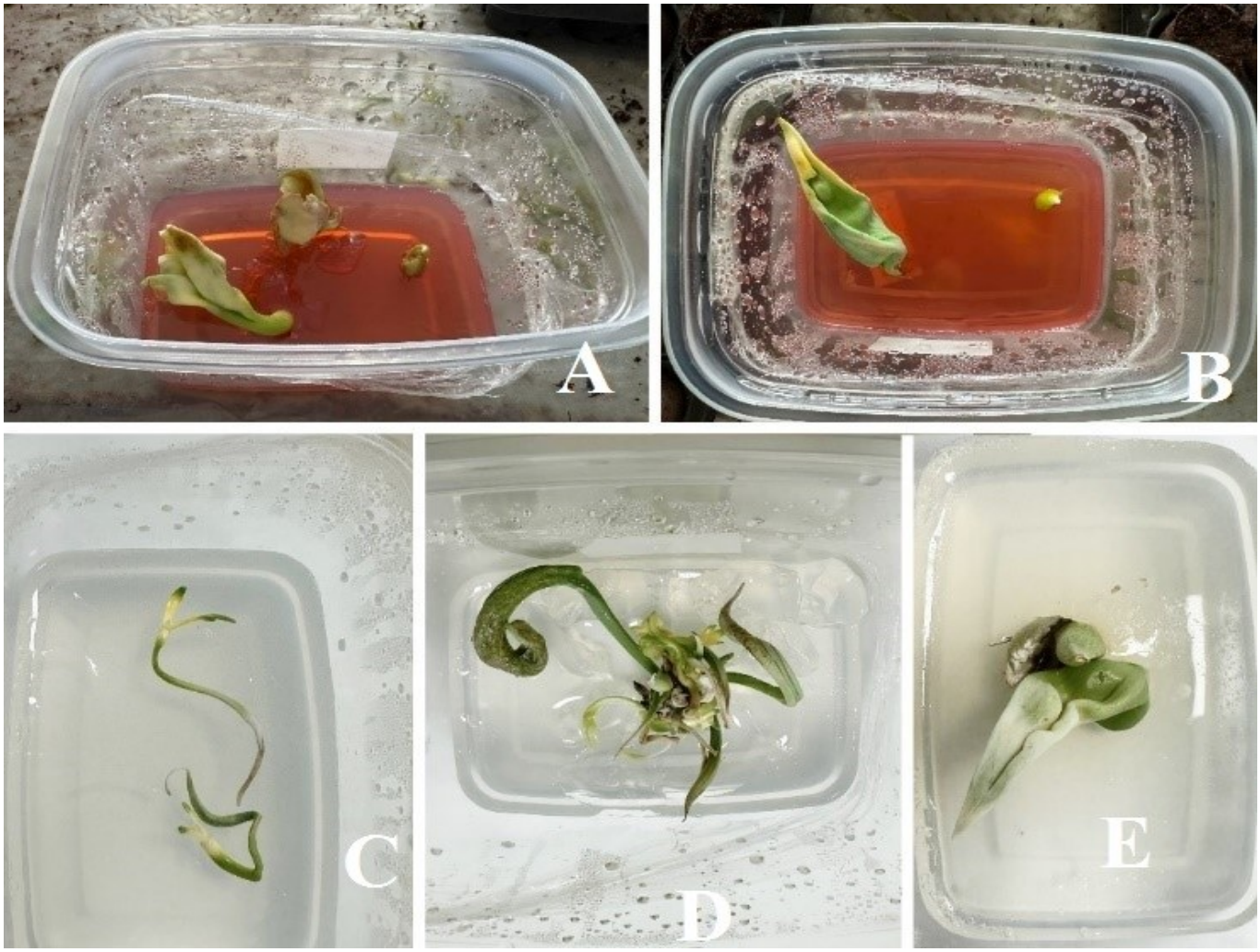

Fig. 6. The growth of seeds and bulbs of *T. fosteriana* and *T. ingens.*: A, B and E is the growth of bulbs, C and D is the growth of seeds and the formation of micro bulbs.

days.

Fig. 6. Visually illustrates these developmental stages. Notably, the results demonstrated that eight shoots and consequently, 8 microbulbs, were obtained from a single embryo when cultured in the medium containing 50-180 seeds in a capsule.

Adaptation mechanisms in response to cultivation environment

It was discovered that *in vitro* culture can yield up to 8 shoots and correspondingly, 8 microtubers are generated on a single embryo. From 50 to 180 completed seeds are formed in the box of one plant. As a result of the experiments, the yield of micro bulbs from the minimum number of seeds produced over 1.5 years averaged 400, the maximum up to 1440. But considering that at the beginning of the study, after the transfer of seedlings to the adaptation process on day 15, the appearance of mold on the surface of the leaves of the bulbs was detected, the expected forecasts may differ slightly from the desired results. Of the 18 microclones, 13 were healthy and 5 were covered with mold. The affected leaves with seedlings were isolated from the general microadaptation environment into a separate environment, where the growth and development of tulips were observed. The affected parts were not treated, since according to the recommendations of the *in vitro* agronomist of the SAG AGRO "Bog'bon" laboratory, treatment with fungicides or other disinfectants affects the growth rate of the plant during the growing season. In this regard, the affected parts of the plant are cleaned of mold and grown in isolated environments and subjected to natural growth and development. According to the literature, mold forms in those places where the cells have undergone necrosis. The mechanical treatment of this part after drying preserves the plant itself and does not slow down the process of vegetation of the plant.

In some approaches the leaves were sprayed with a 50 % aqueous solution of glycerin or a mixture of paraffin (fat) and diethyl ether throughout the entire acclimatization period (1:1). The use of the approach provided 100 % survival rate of regenerants. In our study, we did not use any of glycerin or a mixture of paraffin (fat) and diethyl since the humidity in the greenhouse is automated and varies between 40 and 60 %. The system

itself provides air humidity depending on seasonal changes. For example, in summer this system works intensively and sprays the air with water in the form of steam, and in winter the intensity is reduced due to the natural humidity of the environment. In addition, according to literature data, 20-30 days after planting, plants should be provided with mineral complexes at a temperature of 24 ˚C. However, such work is not carried out, since the soil itself is rich in all mineral salts and fertilizers. In the future, as regenerants grow and depending on their salt needs, regenerants are impregnated with the necessary vitamins and mineral salts. But if the regenerate grows naturally and qualitatively, then no fertilizers are added to the soil and the whole process is trusted to natural growth.

Discussion

Our research findings regarding the effectiveness of a 5 % Domestos solution for plant material sterilization align with and build upon existing literature in the field. Several studies have explored various sterilization methods and their efficacy in ensuring the successful establishment of cultures *in vitro*. For instance, a study by (35) investigated the sterilization of plant explants using different concentrations of bleach solutions. They found that a 5 % bleach solution was the most effective in eliminating contaminants while minimizing tissue damage. This finding corroborates our results regarding the effectiveness of a 5 % concentration of Domestos.

Furthermore, the sterilization process involving multiple stages, including washing with soapy water, potassium permanganate treatment and rinsing with running water, is a commonly employed technique in tissue culture laboratories. This multi-stage approach helps in thoroughly cleaning the plant material and removing surface contaminants before subjecting them to chemical sterilization. In our study, we supplemented the sterilization process with a combination of chemicals, including fundazol, streptomycin, ethanol, and silver nitrate, to enhance its efficacy. This comprehensive approach is supported by the work demonstrated the synergistic effects of combining multiple sterilizing agents in eliminating microbial contamination (34). The optimal conditions identified in our research, namely treating plant material with Domestos (5 %) for 15 min followed by ethanol (70 %) for 2 min, represent a refinement of existing sterilization protocols. This optimized approach not only ensures high levels of sterilization but also minimizes potential tissue damage, leading to increased explant viability.

In the context of seed viability and growth in soil, our study builds upon previous research, which reported viability rates of 23-39 %, increasing to 60-96 % after cold stratification (36). Cold stratification is a common technique used to break seed dormancy and promote germination in many plant species, including *Tulipa*. By subjecting the seeds to a period of cold treatment at 6 °C for 60-78 days, we observed significantly improved germination rates of 88 % for *T. ingens* and 92 % for *T. fosteriana*. This finding suggests that our stratification protocol was highly effective in enhancing seed germination compared to previous studies. Moreover, we focused on optimizing the culture medium to support the growth and development of plant explants. This involved ensuring sterility through autoclaving, which eliminates microbial contaminants and supplementing the medium with vitamins to provide essential nutrients for plant growth. Specifically, we incorporated 4.5 µM Benzylaminopurine and 5 nM IBA to promote rooting before transferring the explants to the greenhouse. The use of cytokinins like Benzylaminopurine and auxins like IBA is well-documented in plant tissue culture for their roles in stimulating root formation and overall growth (36). Our optimized culture medium contributed to the maintenance of viability in 62.5 % of explants under *in vitro* conditions for 6-7 months, indicating its efficacy in supporting prolonged growth.

Furthermore, our study demonstrates successful micropropagation from seeds, yielding eight shoots and microbulbs from a single embryo. Micropropagation, or tissue culture propagation, offers a rapid and efficient means of propagating plants from small amounts of tissue (38). The ability to produce multiple shoots and microbulbs from a single embryo highlights the potential scalability and efficiency of this technique for propagating *Tulipa* species. The transition of regenerated plants from *in vitro* conditions to non-sterile soil involved careful handling, including root washing and the use of a soil substrate consisting of 70 % peat and 30 % vermiculite. This substrate composition provides an ideal balance of water retention and aeration, which are crucial for supporting root growth and overall plant health during acclimatization. Additionally, maintaining adequate illumination and humidity levels during this transition period is critical for ensuring successful adaptation to the new environment. In comparison to other related studies, our research contributes novel insights into the optimization of seed germination, culture medium formulation and micropropagation techniques specific to *Tulipa* species. While previous studies have investigated aspects of seed germination and tissue culture in *Tulipa*, our study offers a comprehensive approach that integrates multiple strategies to maximize the success of plant regeneration and acclimatization. This holistic approach enhances our understanding of the propagation biology of *Tulipa* species and provides valuable knowledge for conservation and cultivation efforts.

The significance of *T. ingens* and *T. fosteriana*, both of which are endangered and rare *Tulipa* species, cannot be overstated. These species are recognized and listed in the Red Data Book of Uzbekistan (17) and the IUCN Red List of Threatened Species (15). *T. ingens* holds the status of "Vulnerable," while *T. fosteriana* is classified as "Near Threatened" under specific criteria, highlighting the precarious conservation status of these taxa. At the global level, the extent of occurrence (EOO) and area of occupancy (AOO) of these species indicate an "Endangered" status in Uzbekistan. The vulnerability of these species is exacerbated by various anthropogenic

impacts, including overgrazing, habitat loss and habitat fragmentation. Additionally, over the past 2 decades, there has been a concerning trend of overexploitation through the collection and commercial trade of their flowers and bulbs. These factors collectively contribute to the decline in population sizes and threaten the long-term survival of these species in their natural habitats. In response to these conservation challenges, our research has implemented a proactive approach by developing micropropagation techniques for *T. ingens* and *T. fosteriana*. By propagating these species *in vitro*, we aim to mitigate the pressures faced by wild populations and contribute to their conservation. Micropropagation offers several advantages over traditional propagation methods, including the rapid production of large numbers of genetically identical plants from small amounts of plant material, thereby reducing the need for wild collection and minimizing genetic variability loss.

Furthermore, our study highlights the effectiveness of sterilization techniques, the optimization of culture medium and adaptation strategies to non-sterile conditions, all of which are crucial aspects of successful micropropagation protocols. By systematically refining these methodologies, we have demonstrated the feasibility of *ex-situ* conservation measures for red-listed *Tulipa* species. Comparatively, other studies in the field of plant conservation have also explored micropropagation as a tool for preserving endangered species. For instance, research focused on the micropropagation of rare orchid species endemic to South America (38). Similarly, a study investigated the use of tissue culture techniques for propagating threatened cactus species in North America (39,40). These studies underscore the broader applicability of micropropagation in conservation efforts across diverse plant taxa and geographical regions.

Conclusion

In conclusion, our research contributes to the conservation of *T. ingens* and *T. fosteriana* by providing practical solutions for their propagation and cultivation. By addressing the threats faced by these species and offering viable conservation strategies, our findings serve as valuable data for future *ex-situ* conservation initiatives aimed at safeguarding red-listed *Tulipa* species from extinction.

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Authors' contributions

SHR contributed to the writing of the article and submission to the journal; KB participated in a survey of laboratory work and gave recommendations on the reproduction of tulips; DD contributed to the design and writing of the manuscript; GM helped in determining the location of plant materials in nature; KX organized a work plan on tulips; MK provided financial support for the work; KT adjusted the style of the article; UZ provided practical assistance in the execution of the work; FR provided assistance in technical equipment; AR helped to ensure that the study complies with ethical standards and legislation; MS conducted a preliminary review of the article before submitting it to the journal.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None.

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