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Micropropagation of Wild Relatives of Cultivated *Tulipa* Species from Samarkand Region (Uzbekistan)

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Abstract *Tulipa* is bulbous geophytes with highly ornamental value worldwide. Anthropogenic impact to the species led to decrease of wild *Tulipa* population including Uzbekistan. The impact challenges development of perspective conservation approaches. Thus, current work presents micropropagation of rare and endangered species *T. fosteriana and T. ingens* distributed in Samarkand region. The study presents optimization of sterilization plant materials, culture media, and adaptation of newly regenerated plants by the use of bulb and seeds of selected taxa.

Keywords Tulipa, in vitro, Microclonal propagation, Culture medium, Bulb propagation, Seed propagation, Germ culture, Growth hormones, Adaptation of regenerants, Soil substrate

1. Introduction

Tulips (*Tulipa* L.) own considerable ornamental and aesthetic value worldwide. At the current time number of species of the genus is unknown due to difficulties in the taxonomy of the genus, confusion in the classifications, high rate of interspecific hybridization and polymorphism (Christenhusz et al., 2013). According to World Flora Online (2023), 376 scientific names related to *Tulipa* are presented and out of 85 (23%) accepted species, 266 (71%) synonymized taxa and 25 (7%) names have not been assessed yet. Currently, the primary center of diversity of wild *Tulipa* presents over 65 species (Dekhkonov et al., 2022a; Asatulloyev et al., 2023;), of which 33 species occur in Uzbekistan (Tojibaev et al., 2022). Despite the long history of *Tulipa* research in Uzbekistan on morphology (Botschantzeva, 1962; Vvedensky & Kovalevskaja, 1971; Dekhkonov et al., 2022a; Everett, 2013; Shukrullo qizi, 2023;), phytogeography (Tojibaev and Beshko, 2014; Dekhkonov et al., 2021;), states of the species under climate change (Dekhkonov et al., 2022b; Asatulloev et al., 2022), molecular studies (Christenhusz et al., 2013; Wilson, 2023), micropropagation of the species has not been studied (Shukrullozoda, 2022a; Shukrullozoda et al, 2022b) enough yet.

The IUCN Red List includes more than 42,100 species threatened with extinction (IUCN, 2023). The rapid growth of the population (Statistics Agency under the President of the Republic of Uzbekistan, 2023) and economy lead to an over-exploitation of natural capital, an increase of urbanization rate and fragmentation/loss of natural habitats. Indeed, 187 species (60%) out of the 314 red-listed taxa of the flora (7.13%) require special protection measures or are not protected at all. Currently, 19 species of *Tulipa* included in Red Data Book of Uzbekistan (Khasanov, 2019) including *T. fosteriana* and *T. ingens*. Main threats of these species are overgrazing, land use for agricultural purposes and overexploitation. One of the effective conservation approaches is micropropagation of plant species (Dekhkonov et al. 2023; Shukrullozoda, 2023;).

in vitro tissue culture techniques are considered one of the effective approaches of conservation of rare and endangered species. There are sufficient contributions on scientific works of Wright N.A. и Alderson P.G. (1980); Gabryszewska, E. And Saniewski, M. (1982); Alderson et al. (1983); B. Aubert, G. Weber and N. Dorion (1985); Nishiuchi Y. (1986); Hulscher M et al. (1992); Kuijpers and Langens-Gerrits (1997); Y.D. Sharma and Shiv Bhushan Kanwar (2003); Minas (2007); Ахметова А.Ш. (2009); Musadiq Hussain Bhat et al. (2020); Podwyszyńska and Marasek-Ciolakowska (2020) *in vitro* micropropagation of *Tulipa* species. However, *in vitro*

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micropropagation of wild relatives of cultivated *Tulipa* species distributed in Uzbekistan has not been developed.

Hence, **the purpose of the work** is to develop micropropagation of wild relatives of ornamental and red-listed species of *T. ingens* and *T. fosteriana* as the effective conservation effort.

2. Material and Methods

Study area. Samarkand is one of the oldest regions of the Republic of Uzbekistan located at the southeast of the country with 123.8 km^2 total area. The region bordered Kashkadaryo (in the south), Bukhara (in the west), Djizzakh (in the east) and Navoi (in the northwest) regions. In terms of biogeography Samarkand is located on the western part of the Pamir-Alay mountains, in the middle of the Zarafshan river. The area is surrounded by Nurata and Aktau mountains of the Turkestan mountain ranges from the north and Zarafshan mountain ranges from the south. The climate is continental. Average annual precipitation is about 282-460 mm.

Plant material and sterilization. Bulbs and seeds of *T. fosteriana* and *T. ingens* were collected from the vicinities Omonquton and Nurabad district.

Sterilization of the bulbs were sterilized by Wright et al. (1980) (3), and Zaitseva (2015) (Zaitseva 2015) which are modified through the study. One bulb of the selected taxa was divided into 2-4 part for the direct formation of callus. Purified and sterilized seed was incubated in optimized culture medium.

Sterilization of plant materials. Optimization of the sterilization of plant materials conducted at "*in vitro* Sag Agro Bog'bon" laboratory (Samarkand, Uzbekistan). Selection and optimization of sterilization agents were presented in table 1.

Table 1. Optimization sterilizing agents and their concentrations

№	Sterilizing agents	Concentration (gr/l)	Effectivity (%)
1	Domestos	5%	90,8
2	Domestos	10%	88
3	Domestos	15%	85
4	Domestos	20%	83,6
5	1. Ethanol 2. Diacid 3. Chloramine	70% 0,1% 0,2%	75,6-78,1

Preparation of the plant materials for the disinfection included following stages: 1) washing seeds and bulbs in soapy water during 20 min at room temperature 2) washing seeds and bulbs in $KMnO_4$ during 15 min at room temperature 3) running water during 50-60 min at room temperature.

Sterilization of plant samples carried out using following complex of chemicals: domestos solution (5%); fundazol (1,20 gr/l, as a fungicide); streptomycin (1 gr/l, as a bactericide); ethanol – 70%; silver nitrate – 0.5%. The sequence of the process presented in table 2.

Table 2. Sterilization sequence of the bulb and seeds of *T. fosteriana* and *T. ingens*

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

To increase the effectivity of the sterilization process, we added silver nitrate with hydrogen peroxide solution and some antimicrobial substances.

Taxonomy. Each species was identified based on the accepted species names according to Zonneveld (2009) and Tojibaev and Beshko (2014).

Culture medium. Optimization the culture is by Murashige and Skoog (1962) was used and was optimized during the work.

Other physical criteria. Micropropagation of bulbs carried out in automatized growing chambers during 16 hours in light (intensity 4-5 K Lux) and 8 hours in dark conditions. Temperature of the cameras were at 26℃ and humidity was between 40-60%.

Optimization of culture medium

Composition of culture medium is one of the important factors in morphogenesis of plant materials. Usually the medium contains macro and micro nutrients, hormones, vitamins, growth regulators and other organic compounds. The media were autoclaved at 121℃ for 5 minutes to dissolve the agar and either 300 ml aliquots were dispensed into plastic container by Duchefa Biochemie company. The culture vessels containing the media were then sterilized by autoclaving for a further 15 min at 121℃. In the first experiment explant of scale, bulbs and seeds were placed on the basic medium supplemented with vitamins: B1, B6, B8

and PP (Table 3).

Table 3. The composition of the nutrient medium for micropropagation *T. fosteriana* and *T. ingens*

Component	Quantity, mg/l	Component	Quantity, mg/l
NH_4NO_3	1650	$Fe_2SO_4 \cdot 7H_2O$	27,95
KNO_3	1880	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	Benzoaminopurine	0.5
$MnSO_4 \cdot 5H_2O$	22,3	Nicotinic acid	100
$CoCl_2 \cdot 6H_2O$	0,025	Mezo-inozitol	6.5-7
$ZnSO_4 \cdot 7H_2O$	8,6	Agar-Agar	2
$CuSO_4 \cdot 5H_2O$	0,025	Hydrolysate	2
Vitamin B1	0,1	Glycine	
Vitamin B6	0,5	Vitamin B8	
Vitamin PP	0,5		

Using this content, we obtained sterile explants of the first generation from the bulbs and seeds. The offspring kept in DKW ((Driver & Kuniyuki Walnut)) medium during 3 months for the propagation of shoots from nodal explants. 4.5 mkm Benzoaminopurine and 5 nM IBA (Indole -3-butyric acid) were included in the medium for the quick development of rooting process before the transferring to the greenhouse. The content of the medium was presented in table 4.

Table 4. Composition of DKW medium for propagation of first generation seedlings

Component	Quantity, mg/l	Component	Quantity, mg/l
NH_4NO_3	1415	H_3BO_3	4.5
Ca $(NO_3 \cdot 4H_2O)$	1960	Na_2MoO_4	0.40
Zn $(NO_3)_2 \cdot H_2O$	17	$FeSO_4 \cdot 7H_2O$	33.60
K_2SO_4	1560	Na_2ЭДТА	45
$MgSO_4 \cdot 7H_2O$	740	Tiamin HCl	2
KH_2PO_4	265	Benzoaminopurine	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

The planting of the explants (bulb) was carried out in the sterile condition at 25℃ and incubated for 3 months in optimized conditions. During the incubation time development of callus of explants observed visually in almost all plant materials (fig. 1).

The next step was micropropagation of selected taxa by the use of seeds. According to literature reviews (Axmetova, 2009), low temperature (from 0℃ to 10℃) causes relief of physiological dormancy of the embryo which induces relatively rapid intraseminal growth. In our case, seed of *T. fosteriana* begun to grow after 80-120 days at 0-10℃ and the growth of the seeds of *T. ingens* grew earlier (78-110 days). It can be explained that tulip seeds are characterized by a deep morphophysiological type of dormancy. The reason for this dormancy is the underdevelopment of the embryo and a strong physiological mechanism for inhibiting germination.

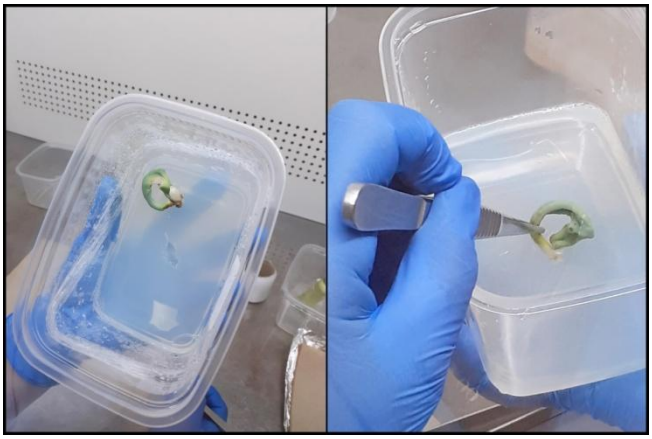

Figure 1. Development of callus and organogenesis from bulb slices of *Tulipa* species by the use of Benzoaminopurine (0.5 mg/l)

Adaptation of regenerated shoots to soil conditions. One of the important and laborious stages of microclonal propagation on which success and effectivity of the process directly depends on it. In the rooting process and planting them in the ground, the basic composition of the medium can be changed by reducing the amount of salts and carbohydrates and quantitative control of plant hormones (elimination of cytokinin and addition of auxin).

Optimal concentration of auxin for the effective shoot growth is important and depends on some reasons. Among them, the hereditary predisposition of shoots to rooting, due to the species and varietal characteristics of the parent plants, the type of auxin used, as well as the concentration and ratio of phytohormones at the stage of shoot propagation. Rooting effectivity of auxin decreases considerable in the presence of high dose of cytokinin in the first and second stages of micropropagation. High concentration of cytokinin in the cultivation process is recommended and lower concentrations of the hormone in further stages gradually (Timofeeva O.A., 2012).

The size of the micro bulb plays crucial role is the transfer of regenerated plants to non-sterile conditions. It has been experimentally shown (Mukhametvafina A., 2009), that the optimal size of micro bulbs in adaptation process to soil conditions is 0.6-0.8 cm in diameter approximately. In our experiment the size of adapting micro bulbs were 1,4 cm – 2,4 cm which corresponded to the above-mentioned data.

In our study, transplantation of regenerated plants into non-sterile conditions or inclusion of micro clones in the adaptation process under *ex-vivo* conditions, involved following stages: a) completely regenerated plants were taken out from disposable sterile containers carefully and b) the root system with leaves is washed in a special solution of polyextradin + phosphoric acid. This solution has a

disinfecting property against fungi and microbes before planting microclones in the soil substrate. The soil substrate previously sterilized at 85-90℃ for 1-2 hours.

In some cases (Timofeeva O.A., 2012), for transformation of regenerants to soil substrate following ratio of substrates were used: peat:sand (3:1); peat:turf soil:perlite (1:1:1); peat:sand:perlite (1:1:1). In our study we required soil consisting of 70% peat and 30% vermiculite was found to be optimal for optimal adaptation medium, for the selected species (fig. 2).

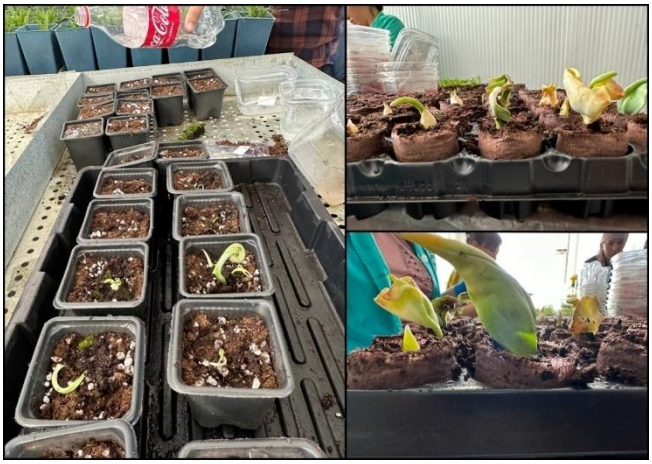

Figure 2. Adaptation of rooting of shoots to soil conditions

The optimal period of the plantation of regenerants is the period when the regenerant possesses growing root system and developing and healthy green leaves which provide fully autonomy of the plant. Noticeable, before the plantation of the regenerants in the selected substrate, the illumination is usually increased to 10,000 lux. Maintained high illumination after the plantation can cause chlorosis of the plants.

It is known, to create relatively high air humidity in the acclimating regenerated plants in the first days is the most important and plays a crucial role in the adaptation of plants and we recommend to reach the relative humidity is about 40-60% which we used during the study. Because, significant water losses at the first days of plantation can lead to the death of micro clones by the high transpiration activity of the leaves and the low absorption capacity of the roots. To increase the survival rate of planted plants in the ground, increase of open-air adaptation time which we recommend 2-3 hours in a week. For the prevention of rapid dehydration of the leaves grown *in vitro* during their transplantation some surface-active organic substances can be applied which reduces transpiration rate considerably.

3. Results

According to the results, sterilization of the plant materials with 5% concentration of Domestos found to be the most effective and applied in further studies.

Totally, the sterilization sequence contained 9 stages which included 5 times of treatment with fungicides and bactericides. Regime for isolated samples from was conducted depending on the type of explant. The presented sterilization approach of the seed and bulb slices of *Tulipa* showed as a perspective method for the scale up processes.

Three criteria for the identification of the effectivity of the sterilization process were selected: number of infected (1) and necrotic (2) of planting samples and number of viable explants (3) after the sterilization process. Due to results, maximal number of viable explants (%), minimal infected (%) and necrotic (%) explants were obtained after the treatment of the bulb slices Domestos (5%) during 15 min and in the ethanol (70%) during 2 min. The effective optimization approach for the bulb sterilization allowed to obtain high percentage of viable explants.

Viability of seeds and growth rate in the ground is about 23-39% (Botschantseva, 1962) and the rate was increased up to 60-96% after cold stratification process. In our study, stratification of seeds conducted at 6°C during 60-78 days. In this condition seeds of *T. ingens* and *T. fosteriana* begun to germinate after 35 and 41 days respectively. The growth effectivity of seeds was high for both species (*T. ingens* – 88% and *T. fosteriana* – 92%).

After 6-7 months of cultivation 62.5% of explants were viable. Under *in vitro* conditions, each tulip shoot has an apical meristem surrounded by the base of a single curled leaf. After cooling process intensive growth of the leaves was observed. The first morphologic character in the bulb formation process is cessation of leaf growth, swelling of the bases of shoots and their progressive yellowing (Novak et al., 2014).

Thus, in our study, seed growth was observed after 116 days, and the visible appearance of seed sprouts could be seen after 131 days (fig. 3.). Our results showed that eight shoots and accordingly, eight microbulbs were obtained from one embryo in the culture medium with 50-180 seeds in a capsule.

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℃. 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.

Figure 3. The growth of seeds of *T. fosteriana* and *T. ingens*

4. Discussion

Podwyszyńska in their research work (2020) used a method of somatic embryogenesis. This method for tulip regeneration via somatic embryogenesis (SE) was developed using flower stem slices from cooled bulbs. These slices were cultured in darkness on modified MS media with auxins (2,4-D, NAA, and picloram) alone or with TDZ at 0.1 and 0.5 mg L−1. Callus formation occurred on the incised surface, leading to embryogenic callus lines. TDZ enhanced somatic embryo production, with the best results obtained using 2,4-D at 0.1 mg L−1 and TDZ at 0.5 mg L−1. Proline addition improved callus proliferation or embryo formation. High-quality embryos, capable of bulb formation, were observed with BAP at 0.1 mg L−1 instead of TDZ (7).

The study investigates a sterilization method for Tulipa seeds and bulb slices using a 5% Domestos solution, which proved to be the most effective among tested protocols. This method, comprising nine stages including five treatments with fungicides and bactericides, is highlighted for its potential in scaling up processes. Sterilization effectiveness was gauged through three criteria: the number of infected and necrotic samples and the number of viable explants post-sterilization, where treatment with Domestos followed by ethanol significantly increased the viability of explants. Additionally, cold stratification of seeds at 6℃ for 60-78 days markedly improved growth rates for T. ingens and T. fosteriana, showing an increase in seed viability up to 60-96%.

Furthermore, the study explores in vitro cultivation outcomes, where a 62.5% viability rate was noted post-cultivation, alongside the development of morphological indicators of healthy growth. The research also reports on the germination and growth processes, where seeds showed visible sprouts after 131 days, leading to successful shoot and microbulb formation. This study suggests the efficiency of the sterilization process and cold stratification in enhancing the viability and growth of Tulipa, potentially applicable to other plant species. The innovative approach of relying on automated greenhouse humidity control further emphasizes the possibility of minimizing external intervention, leaning towards a more natural growth process.

For a detailed analysis of the germination of the studied species in the external environment, further studies will be required to ensure the availability of wild species for germplasm collections.

5. Conclusions

Regarding seed viability and growth in soil, previous research (Botschantseva, 1962) reported viability rates of 23-39%, increasing to 60-96% after cold stratification. In our study, we conducted seed stratification at 6℃ for 60-78 days, resulting in germination rates of 88% for *T. ingens* and 92% for *T. fosteriana*.

We also optimized the culture medium, ensuring sterility through autoclaving and adding vitamins. The medium contained 4.5 µM Benzylaminopurine and 5 nM IBA for rooting before transferring to the greenhouse. After 6-7 months, 62.5% of explants remained viable under in vitro conditions. Micropropagation from seeds yielded successful growth, with eight shoots and microbulbs from one embryo.

The transition of regenerated plants to non-sterile soil involved careful steps, including root washing and using a soil substrate of 70% peat and 30% vermiculite. Adequate illumination and humidity during acclimatization were critical for adaptation.

Sterilization of the plant materials, optimization of culture medium, *in vitro* propagation protocols and adaptation techniques were developed for the selected taxa. We believe that our results will serve as a valuable data for *ex situ* conservation measure some red-listed *Tulipa* species in the future.

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