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Scientific Article

The application of different reproduction techniques for rare species waterlily tulip (*Tulipa kaufmanniana* Regel.) propagation under *ex situ* conditions

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Abstract

T. kaufmanniana is a threatened and endemic species of the Tien Shan Mountains with a complex of valuable ornamental features. However, the commercial usage of the plant is limited due to the restricted availability of the bulbs in nature, difficulties in overcoming seed dormancy and low efficiency of the species reproduction. Results of the investigation of the reproductive biology of this species in the culture conditions allowed us to characterize *T. kaufmanniana* as viable with successful seedage. In the work reported here the effect of low temperature on the proper development of embryos is addressed, and an attempt is made to clarify the effect of cytokinins (TDZ, BAP) and auxin (NAA) on the regeneration capacity of isolated *T. kaufmanniana* embryos for the adventitious shoots and bulblets formation. Direct shoot organogenesis was induced by the combined action of chilling and BAP or TDZ treatment. Among the variants tested, TDZ promoted the formation of adventitious bulbs in 48 % of the chilled embryos, compared to non-chilled embryos (0.0%) and embryos exposed to the same concentration of BAP (39%). The best culture condition for *T. kaufmanniana* embryos growth and bulblets formation consisted of chilling for 10 wk in the dark at 4 °C, 4 wk at 20 °C under 16 h light photoperiod and 10 wk at 4 °C in the dark. Sufficiently effective protocol for clonal micropropagation was developed for the first time for *T. kaufmanniana* by the use of immature zygotic embryos as primary explants.

Keywords: *Tulipa* species, seed production, embryo culture, immature zygotic embryos, chilling, bulblet formation, growth regulators

Introduction

Representatives of the *Tulipa* L. genus are of special interest for studies because they are spring xerophytic bulbous ephemeroid plants with very short vegetation period and with pronounced reaction to environmental changes (Khodorova and Boitel-Conti, 2013).

The *Tulipa* L. genus includes 83 species according to Botschantzeva and Varekamp (1982) and 76 species according to Christenhusz et al. (2013); they spread across the regions of Europe, Asia and Northern Africa with temperate climate. The largest number of species concentrated in Middle Asia (Kazakhstan, Kyrgyzstan, Uzbekistan and Turkmenistan) was evaluated as 63 (Botschantzeva and Varekamp, 1982) or as 46 species, according to Christenhusz with co-workers (2013). *T. kaufmanniana* is among threatened and endemic species originated in Tien Shan and Pamir-Alai mountainous regions due to isolation (The Red Data Book, 2019). Tulip species have been studied *in situ* mainly for the purpose of their protection, while under *ex situ* conditions tulip species as the source of a large number of varieties have been studied for the purpose of introducing them into culture and breeding. At present, there is only scarce information about the adaptation potential of tulip species, which can be useful for the development and introduction of populations (Voronin, 1987; Kashin et al., 2016).

T. kaufmanniana is a valuable plant species with ornamental potential: it is one of the species with the earliest flowering, which can re-flower without being replanted (Botschantzeva and Varekamp, 1982). The species gives rise to an independent cultivar group and has an urgent need for mass propagation. However, there are two bottleneck aspects concerning the species propagation: the limited amount of annual daughter bulbs formation (Kudryavtseva, 1987) and a deep complex morphophysiological type of seeds dormancy (Nikolaeva et al., 1999). All tulips are commercially propagated through asexual reproduction by using bulbs, but the efficiency of this process is low (Kamenetsky and Okubo, 2013). As an alternative to the

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conventional methods, biotechnology techniques make it possible to shorten the time needed to cultivate tulips and to obtain large numbers of vigorous plants with high quality and free of endogenous pathogens (Podwyszynska and Marasek, 2003; Sochacki and Podwyszynska, 2012). The *in vitro* techniques appear to be efficient for the conservation of tulip genetic resources only if the genetic stability of regenerated plants is approved by molecular markers (Kritskaya et al., 2019).

Such explants as seed, bulbs and scales are commonly used for tulip micropropagation (Orlikowska et al., 2018). However, it was indicated that the seeds of *T. kaufmanniana* did not germinate without pre-chilling, and only after 5 or 7 weeks of stratification the germination rate was enhanced significantly (ROUHI et al., 2010), whereas the authors who used isolated embryos as primary explants for the propagation of tulips from Kaufmanniana group reported about a high frequency of bulb formation *via* adventitious bud regeneration (Akhmetova et al., 2011) or somatic embryogenesis (Timina et al., 2016). So it seems that immature and mature embryos or any parts of floral buds are the most suitable explants for *in vitro* regeneration of tulips and other bulbous plants (Ziv and Lilien-Kipnis, 2000; Uzun et al., 2014; Timina et al., 2016), because these explants are known to be rich in meristematic tissues, which remain genetically stable during multiple subculturing (Butenko, 1999).

Recently embryo culture has been developed not only to overcome interspecific incompatibility of tulips (Van Creij et al., 1999), but also as the method for rapid propagation of hybrid tulip varieties (Akhmetova et al., 2011) and *Fritillaria* species (Muraseva and Novikova, 2018). It is known that after pollination of a tulip mother plant, a zygote develops into a mature embryo within a period of 12 weeks (Van Tuyl and Van Creij, 2006). It was reported that the optimal viability of hybrid tulip embryos was the highest when excised seven weeks (Custers et al., 1995), or at 53-56 days after pollination (Akhmetova et al., 2011). Hence, there was the necessity to find an appropriate stage of *T. kaufmanniana* embryo development and the degree of its differentiation for the excision and its further cultivation *in vitro*.

One of the most important factors affecting the growth and development of tulip is temperature. The tulip has an obligatory warm-cool-warm cycle (De Hertogh and Le Nard, 1993). A period of low temperature is required to interrupt the embryo dormancy, induce germination and initiate bulb primordium (Khodorova and Boitel-Conti, 2013). Furthermore, it was reported that the *in vitro* formation of bulblets from tulip was induced by cultivation at 20°C for 14 to 18 wk (Maslanka and Bach, 2014), while in another study the regenerated shoots were able to form bulblets only after cold treatment for 12 wk (Kuijpers and Langens-Gerrits, 1997).

During the recent decades, several studies testing the regeneration response of different tulip explants to exogenous treatment including hormones, different temperature and illumination regimes have been carried out (Custers et al., 1992; Van Rossum et al., 1997; Podwyszynska et al., 2014; Orlikowska et al., 2018).

Although conditions suitable for bulb formation in *vitro* have been in common preliminarily defined for many tulip explants and genotypes, this process remains inefficient for obtaining a sufficient number of bulbs in *T. kaufmanniana* embryo culture.

The aim of this work was to study the reproductive biology of *T. kaufmanniana* under the conditions of West Siberia during the species introduction either by seeds or embryo culture in order to develop the strategy of the species conservation *ex vivo*. The program of *T. kaufmanniana* reproduction involved conventional breeding methods along with biotechnological ones. So, in the present study it was necessary to optimize the protocols for enhanced vegetative propagation in the embryo culture of *T. kaufmanniana* and to study the initial morphogenetic reactions of the tulip embryos to selected kinds of treatment (temperature, illumination conditions, phytohormones). The use of the embryo culture makes it possible to increase the rate of *T. kaufmanniana* reproduction and to elucidate the regulation of immature embryo development.

Materials and Methods

Plant material

The object of investigation was *T. kaufmanniana* – an endemic species of the Western Tien Shan (The Red Data Book, 2019). According to the modern classification (Christenhusz et al., 2013), *T. kaufmanniana* belongs to the *Tulipa* subgenus and is distinguished by intraspecific diversity (Figure 1A-B).

Figure 1. A and B – polymorphism of the *T. kaufmanniana* perianth coloration among plants from the introduction population.

For this study the bulbs of flowering plants were collected during a field survey in 2009 in the Namangan Region of the Republic of Uzbekistan. This tulip species has been grown outdoors for 9 years under the natural conditions of the Central Siberian Botanical Garden. Meanwhile, the natural habitats of *T. kaufmanniana* are substantially different in terms of climatic conditions from the region of species introduction – Novosibirsk Region (Aizenshtat et al., 1984; Weather and Climate) (Table 1).

Table 1. Comparison between the climatic parameters of the regions of collection and introduction.

Climatic characteristics	Sites of collection (Namangan region)	Site of introduction (Novosibirsk, CSBG)
Climate	subtropical	continental
Average annual temperature (T °)	+14.8	+1.8
Precipitation (mm/year)	400	420
Air humidity (%)	56	76
Snow cover (cm)	50	70

Normally this tulip species can cross-pollinate, but hybridization with other species is impossible because of its relatively early flowering. To study the seed productivity and reproduction of this species, we used the seeds obtained from the free open pollination of the introduced plants. The total number of seeds was 396 (minimum) – 2101 (maximum) in the evaluation of seed productivity and in seed planting.

Tissue culture material and procedures

T. kaufmanniana immature seeds were harvested in June, 2017 from fruits 2.8-4.0 mm long, within 50-54 DAA. It is well known that only one or two days after anthesis, the stigma is receptive and the flowers can be pollinated (Van Creij et al., 1999), so we believe that the time of anthesis is a suitable indicator in the case of natural pollination of *T. kaufmanniana*. As the excision and cultivation of globular tulip embryos is difficult, similarly to nearly mature ones, we used the embryos at torpedo stage with distinct cotyledon (Figure 2) at the age of 52-54 DAA, which were found to be autonomous in their development *in vitro*.

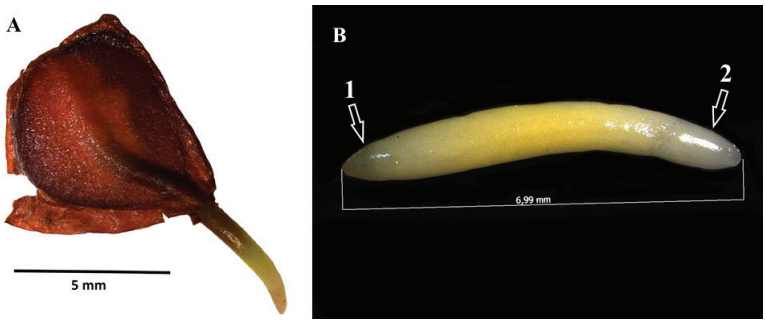

Figure 2. Initial stages of seed reproduction of *T. kaufmanniana* outdoors: A – germinating mature seed; B – differentiation of organs in the seed embryo: 1 – haustorium, 2 – root.

Seed capsules were surface-disinfected with 70% ethanol for 1 min, followed by immersion in a 15% solution of Domestos for 15 min and rinsing 3 times with sterile water. Zygotic embryos were isolated from seeds aseptically under a binocular microscope; these embryos were taken as the explants. Embryos were placed flat halfway embedded on the MS medium (Murashige and Skoog, 1962) without PGRs or supplemented with 0.3 mg L^{-1} NAA, 0.5 mg L^{-1} BAP or TDZ. Six different treatments were used for *T. kaufmanniana* embryo culture (Table 2).

Table 2. Different treatments used for the *in vitro* cultivation of *T. kaufmanniana* embryos.

Media variants	Additives to MS medium	Cultivation conditions	Incubation period
Variant 1 (control 1)	-	4°C (D*)	24 wk
Variant 1A	0.5 mg L^{-1} BAP+ 0.3 mg L^{-1} NAA	4°C/20°C/4°C (D/L/D)	10 wk/4 wk/10 wk
Variant 1B	0.5 mg L^{-1} TDZ+ 0.3 mg L^{-1} NAA	4°C/20°C/4°C (D/L/D)	10 wk/4 wk/10 wk
Variant 2 (control 2)	-	20°C (L*)	24 wk
Variant 2A	0.5 mg L^{-1} BAP+ 0.3 mg L^{-1} NAA	20°C/4°C/20°C (L/D/L)	7 wk/10 wk/7 wk
Variant 2B	0.5 mg L^{-1} TDZ+ 0.3 mg L^{-1} NAA	20°C/4°C/20°C (L/D/L)	7 wk/10 wk/7 wk

*D - in the dark; L - in the light.

In every treatment 30 embryos of the same age and size were excised and 3 to 5 embryos placed in a 150 ml flask containing 30 ml medium. All the experiments were repeated twice. Half of all embryos were chilled for 10 wk at 4 °C in darkness, while the remaining embryos were exposed to 20 °C and photoperiod of 16 h of daylight under fluorescent lamps (30 μmol m^{-2} s^{-1}) for the same period of time. The morphology of the embryos and sprouting shoots was observed at the time of excision, 7 wk and 24 wk after the start of the experiment.

The cultures were maintained at media either lacking PGRs under continuous temperature regime (Variants 1, 2) or containing PGRs under changing temperatures (Variants 1A, 1B and Variants 2A, 2B). After this period, the chilled embryos were placed in the warm conditions and non-chilled – in the cold ones and then returned to the same conditions for the last period for bulblet initiation. Embryo cultures kept under unchanged conditions (control 1 – 24 wk at 4 °C in the dark; control 2 – 24 wk at 20 °C in the light) were taken as a control. Then, after 24 wk, the regenerated microplants were transferred for multiplication on the MS medium containing different concentrations of BAP from 0.1 to 0.3 mg L^{-1}. For rooting, adventitious shoots were maintained on the MS medium with 0.1% of activated charcoal and no PGRs.

Methods

The seed productivity of mature fruit was studied using the procedure proposed by Vainagiy (1974). We determined the PSP (potential seed productivity) as the number of ovules; ASP (actual seed productivity) as the number of seeds; SP (seminification percentage) as the percentage of ovaries, and SP = (ASP/PSP) x 100%. After having calculated the seed productivity, the selected seeds

with viable embryos were sowed in the plots with similar soil conditions during autumn before the formation of the snow cover. Embryos and seedlings were examined using Axioplan 2 imaging (Carl Zeiss) microscope and photographed with the AxioCam MRc5 high-resolution digital camera AxioVision 4.8 with the digital image processing software.

Statistical analysis

Each experimental treatment was repeated twice and for each treatment 30 embryos of the same age and size were used. During seed evaluation, we calculated: V – coefficient of variation (%), r – correlation coefficient, b_{yx} – regression coefficient. The results are expressed as the mean (M) ± standard error of the mean (SE) of at least two independent experiments. The data was analyzed using Microsoft Office Excel 2007 and one-way analysis of variance (ANOVA), followed by Duncan test. The values of $P < 0.05$ were considered significant.

Results and Discussion

In this study we have shown that the *T. kaufmanniana* coefficient of vegetative reproduction after cultivation of bulbs under the conditions of the Novosibirsk Region was equal to 0.9. Hence, due to the observed low coefficient of vegetative reproduction and verification of the formation of viable seeds under the conditions of the Novosibirsk region (Table 3 and Table 4), we carried out the further studies with seeds as starting material.

Table 3. Morphological characteristics of *T. kaufmanniana* seeds.

Seed length (mm)	mature seeds	immature seeds
M±SE	7.36±0.08	6.45
V	11.82	
Embryo length (mm)		
M±SE	3.78±0.08	2.08±0.14
V (%)	10.85	13.59
Regression data of the parameters		
r	0.52	
b_{yx}	0.55	

Table 4. Seed productivity of *T. kaufmanniana* during adaptation.

Year		2013	2015	2016	2017	Average over years
Number of fruits		12	17	5	7	10
Total number of seeds		1488	2101	396	836	1205
PSP	M±SE	255.1±11.6	239.7±9.4	247.0±19.5	250.6±16.6	260.57±26.1
	V	15.5	16.2	17.6	18.9	26.5
ASP	M±SE	124.0±14.9	123.6±12.8	79.2±22.5	111.5±17.9	119.4±21.6
	V	41.6	42.3	63.3	48.7	47.7
SP	min	15.4	24.6	8.3	16.8	18.8
	average	49.6	53.5	30.7	44.5	44.2
	max	82.0	85.1	48.4	70.3	65.8

Studying the relationship between seed length and embryo size in mature seeds of *T. kaufmanniana*, we observed a correlation coefficient of 0.52 between these two parameters and a regression coefficient (b_{yx}), which points to a positive effect of seed length on embryo length.

To evaluate the vitality of *T. kaufmanniana* during adaptation under *ex situ* conditions, we studied the seed productivity (Table 4). We observed an average seminification factor (%) (or the percentage of ovaries) of 45%, which allows us to characterize this species as able to reproduce successfully *via* seeds under the conditions of introduction. In the work concerning the reproduction biology of threatened tulip species, the authors indicated that the seed productivity in natural populations was low: only one-third of the ovules of the Caucasus endemic *T. eichleri* produced fertile seeds (Mikatadze-Pantsulaia et al., 2010).

The reproduction of tulips *via* seeds requires clear compliance with the temperature and light regimes; this is a labor-intensive and long-term process. Different levels of

morphophysiological dormancy are characteristic of tulip seeds. This is caused by insufficient development of the embryo and strong physiological germination inhibition mechanisms (Rouhi et al., 2012). Other authors have shown that the seeds of all tulip species require a long period of low temperature and substantial humidity for germination (Khodorova and Boitel-Conti, 2013).

The ontogenesis of the pregenerative period under the conditions of Tashkent usually lasts for 4 years (Botschantzeva and Varekamp, 1982). When studying the pregenerative period, we carried out experiments since 2013, starting with 1488 seeds of *T. kaufmanniana*. With the seed germination capacity equal to 42.30%, all the 242 plants survived were revealed to be still immature in 2018. An increase in the periods of ontogenesis could be an indicator of the stress reaction during the adaptation of the species to the conditions of the introduction region (Gerasimovich and Vasilyeva, 2017). In this study, in spite of climatic differences, no substantial differences between initial morphogenetic reactions of the tulip seedlings development under the field and *in vitro* conditions were observed on the basis of the morphological parameters measured. But the duration of the whole ontogenetic cycle of *T. kaufmanniana* appeared to be dependent on the changes of the growth conditions during the species introduction in West Siberia.

The pathways of embryo development *in vivo* and *in vitro*

We investigated the early stages of the ontogenesis of *T. kaufmanniana* seedlings grown from seeds planted in soil in the CSBG. We observed that the embryo develops inside the seed forming a haustorium, the main function of which is to absorb and store substances from the endosperm and form the seedling leaf (Figure 2A-B; Figure 3C). While the haustorium grows, the lower part of the embryo expands forming an apical bud, the first rootlet and a dropper the main function of which is primary deepening of the seedling (Figure 3A). During germination, the white-colored haustorium gets liberated from the seed coat and it is followed by almost cylindrical chlorophyll-containing cotyledons. The dropper also grows lengthwise and becomes thicker at the end, where a displaced apical bud of the seedling develops (Figure 3B-D).

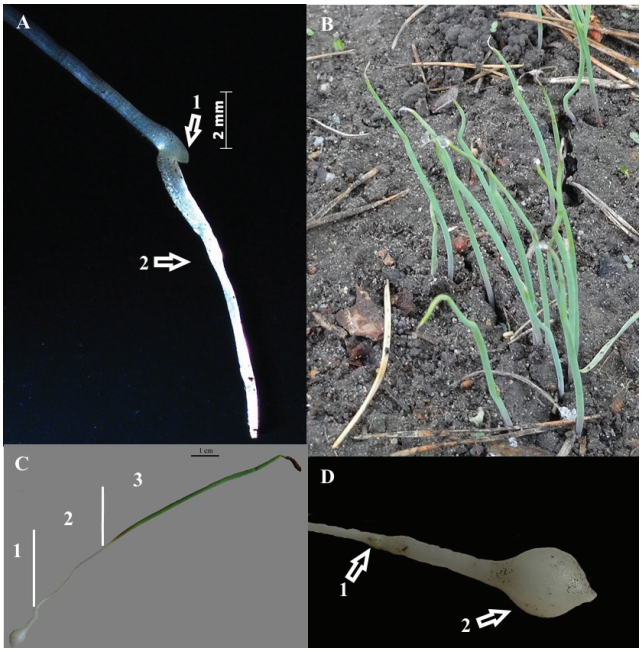

Figure 3. The development of *T. kaufmanniana* seedling: A – seedling (14 days); B – an above-ground part of a seedling, rising after 5 days: 1 – dropper formation, 2 – rootlet; C – seedling (34 days): 1 – dropper, 2 – haustorium, 3 – cotyledon; D – underground part of seedling (34 days): 1 – the site of primary root attachment, 2 – expanded dropper with the displaced apical bud.

Under the conditions of the CSBG, *T. kaufmanniana* seeds planted in September of the previous year germinated in the middle of April at the first week after the snow cover disappeared. Cotyledons extinction occurred in the middle of June (Figure 3B), and the vegetation period lasted for 60 days. For comparison, the duration of the first year seedlings vegetation under the conditions of Tashkent is 51 days for *T. kaufmanniana* and slightly shorter than the one observed in this study (Botschantzeva and Varekamp, 1982).

For the development of immature embryos *in vitro*, it was necessary to determine the optimal conditions for their germination and multiplication. Previous studies have shown that the regeneration capacity of tulips is strongly influenced by the physical culture conditions (Sochacki and Podwyszynska, 2012), the developmental stage and physiological state of the initial explants (Timina et al., 2016) and the culture media composition (Cig and Basdogan, 2015). Niimi (1978) confirmed that

the vegetative apex even in mature tulip embryos is not completely developed. It was shown that 50-53 days old embryos after anthesis were able to further develop into spindle shaped embryos *in vitro* with a basic culture media (Williams et al. 1987). In the present study the optimum period for embryo isolation has been determined for *T. kaufmanniana*. In general, embryos were autonomous in their development *in vitro* at the age of 52-54 DAA. When the embryos were younger at the start of the culture (less than 50 DAA), it was difficult to isolate and maintain them under the optimal culture conditions *in vitro*.

As the next step for obtaining properly developed adventitious bulbs of *T. kaufmanniana*, we tested different combinations of cold incubation and PGRs previously shown to influence the efficiency of embryo *in vitro* culture of many crops (Maslanka and Bach, 2014; Uzun et al., 2014). In our experiment, germination of the embryos cultivated *in vitro* was observed immediately 3 days after excision, when they began to enlarge, develop the radicle and then hypocotyl (Figure 4A-B). Subsequently, the dropper and cotyledon were formed (Figure 4C-D). Properly germinating embryos had a bulb on the seedling base (Figure 4E). Generally, chilled explants formed adventitious bulblets on media containing 0.5 mgL^{-1} TDZ + 0.3 mg L^{-1} NAA kept in the dark (Figure 4E). Our observation confirms previous findings of other studies that the induction of tulip bulblets occurred in darkness after seed cultivation at 5°C during 10-12 wk period (Kuijpers and Langens-Gerrits, 1997).

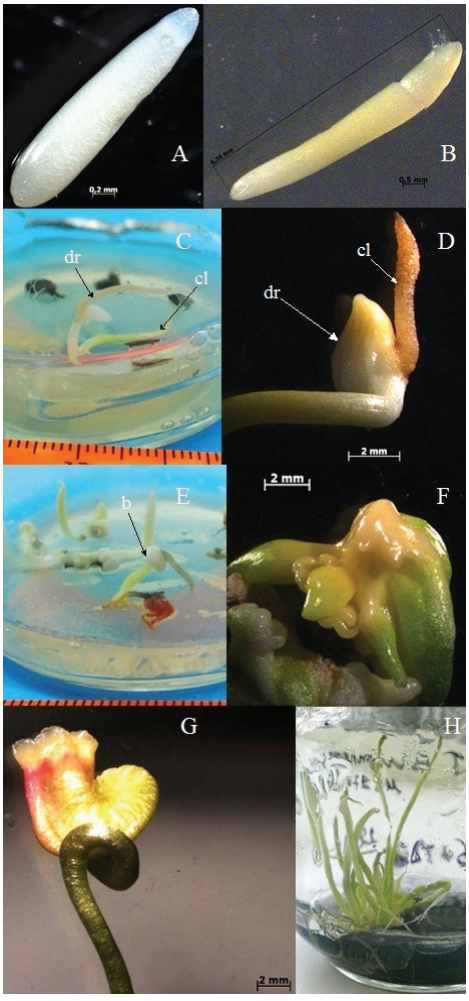

Figure 4. The early stages of immature embryos *T. kaufmanniana* development *in vitro*:
A – embryo at the stage of excision (52-54 DAA); B – differentiation of embryo organs after 10 days of cultivation on Variant 1 medium; C, D – the formation of dropper (dr) and cotyledon leaf (cl); E – the initial stages of bulbs formation (b) on Variant 1B medium after exposure to light and temperature of 20°C followed by chilling at 4°C for 12 wk; F – appearance of fasciated cotyledon leaves developed under influence of high temperature and TDZ addition to the initial medium (Variant 2B); G – haustorium-like region in the base of cotyledon leaf; H – shoot cultures initiated after 8 wk maintenance on multiplication medium.

Subsequently, from the embryos induced to organogenesis (Figure 4H) adventitious shoots developed mainly *via* direct organogenesis and formed clusters during 8 wk maintenance on multiplication MS medium, supplemented with BAP in the concentrations of 0.1-0.3 mg L^{-1}.

Our experiment showed that different concentrations of BAP, TDZ or NAA affected the germination percentages of chilled embryos causing a slight increase. The germination percentage did not differ between variants 2A and 2B, but the addition of cytokinins BAP or TDZ increased the germination percentage of the embryos cultivated at the first stage under cold conditions, as compared with the control (Variants 1, 1A, 1B). Remarkably, the embryos subjected to chilling at 4 °C for 10 wk in the dark, then at 20 °C under 16h light photoperiod for 4 wk and then cultivated at 4 °C for 4 wk in the dark developed a larger number of adventitious buds, and almost half of explants regenerated bulblets (Table 5). In the experiment, TDZ promoted the formation of adventitious bulbs in 48% of the chilled embryos (Table 5), compared to non-chilled embryos (0.0%) and the embryos exposed to the same concentration of BAP (39%). Cytokinins were usually used, either alone or with auxin (Podwyszynska and Sochacki, 2010), to initiate shoot organogenesis in tulips. Explants treated with BAP often formed callus tissue, from which adventitious bulbs differentiated (Maslanka and Bach, 2014). Podwyszynska and Marasek (2003) have described multiplication of shoots, using TDZ instead of the currently used cytokinins – 2iP and BAP. Fragments of flower stems of 6 cultivars, used as initial explants, were incubated for 2 months in darkness on the medium containing NAA and cytokinins, 2iP and BAP, as a control, or TDZ and paclobutrazol. In their study, TDZ – very stable phenylurea compound with strong cytokinin-like activity (Guo et al., 2011) – greatly increased shoot regeneration in comparison with other treatments. These results are not in agreement with the results concerning bulb formation in the *in vitro* cultures of two tulip cultivars (Podwyszynska et al., 2014). The authors approved that the low bulbing capacity of flower stalk chilled explants of two cultivars was associated with the high shoot multiplication ability, affected by the increased endogenous cytokinin status of regenerants, revealed after TDZ treatment (in comparison with 2iP). Based on these findings, their recommendation was to replace TDZ with 2iP in tulip micropropagation in the last multiplication subculture. We suppose that this non-coincidence of the results might be related to the variation in the physiological status of the explants excised from the different tulip genotypes.

Table 5. Effects of different temperatures and cold treatment periods on the germination of *T. kaufmanniana* embryos.

Media variants	Germination rate (%)*	Explants forming callus (%)*	Bulblet regeneration (%)*	Number of bulblets*
Variant 1 (control 1)	80.72±6.67 c	0.03±0.00 a	21.60 ±1.28 c	2.25 ± 0.13 b
Variant 1 A	78.82±3.04 c	2.7±0.30 ab	38.76±4.62 d	3.73±0.07 bc
Variant 1B	74.70±3.33 c	5.5±0.24 b	48.02±2.50 e	4.62±0.06 c
Variant 2 (control 2)	4.55±0.56 a	1.22±0.06 a	0.00±0.00 a	0.00±0.00 a
Variant 2A	29.47±1.80 b	24.33±2.80 c	1.56±0.08 ab	1.80±0.05 ab
Variant 2B	34.58±2.64 b	31.92±2.04 d	8.66±0.43 b	1.64±0.03 ab

*Values in the column followed by different letters are significantly different at the 0.01 level

In the presented study, as in other experiments (Maslanka and Bach, 2014), adventitious shoots appeared on the explants through direct organogenesis (Figure 4H), although shoot differentiation *via* callusogenesis has also been observed. More abundant callus appeared when the explants were non-chilled and cultivation media were supplemented with PGRs. Rarely, embryos in these conditions exhibited some abnormalities of their development (Figure 4F). The *in vitro* culture fasciation is a culture-induced abnormality in plantlets that occurs due to flattening of the shoot, fusion of organs and formation of multiple points of growth, resulting from asynchronic pattern of cell development as described by Iliev and Kitin (2011). It was worth noting that fasciating cotyledonary leaf-like malformed structures occurred mainly in non-chilled explants under the treatment of BAP or TDZ, implemented in the cultivation medium together with NAA (Table 5, Variants 2A, 2B).

The treatment with 0.5 mg L^{-1} BAP or TDZ along with 0.3 mg·L^{-1} NAA resulted in the formation of 4-5 bulblets per explants when temperature and light regimes were subsequently changed (Variants 1A, 1B). No influence of the addition of BAP or TDZ to the media was observed on the number of bulbs formed when embryos were not chilled at first (Variants 2, 2A, 2B).

As the frequencies of bulb regeneration (39-48%) in Variants 1A and 1B were significantly higher in comparison with other treatments, we could indicate that the induction of adventitious bulb formation was caused not only by chilling the explants (22% frequency) but mostly by the changing temperature regime during embryo proliferation. Finally, we could show that the *in vitro* development of

the immature embryos of *T. kaufmanniana* embryos was morphologically similar to mature seeds embryos during germination in soil with the formation of cotyledon, haustorium and dropper structures.

Conclusions

As a result of the long-term introduction experiment, the reproductive potential of *T. kaufmanniana* was studied and comparative analysis was made concerning the morphogenetic reactions of the tulip seedlings development under the field conditions and during the *in vitro* culture observations. The creation of a stable introduced population of *T. kaufmanniana* allowed us to characterize this species as a viable, with successful seed reproduction under the conditions of introduction. The existence of this population and the availability of a sufficient amount of plant material allowed us to carry out experiments focused on the culture of immature embryos and determine factors enhancing the formation of adventitious bulblets *in vitro*. It was shown that the optimal period for embryo isolation was between 52 and 54 days after *T. kaufmanniana* anthesis. Changes in the temperature regime for the *in vitro* tulip embryo culture significantly improved the germination percentage in comparison with the treatments in which the embryos were not chilled. The best embryo growth culture condition consisted of chilling for 10 wk in the dark at 4 °C, 4 wk at 20 °C under 16h light photoperiod and 10 wk at 4 °C in the dark. These conditions were also the most optimal ones for bulblet formation in the development of *T. kaufmanniana* embryos. The efficiency of *in vitro* embryo culture methods proved to be dependent on the type of cytokinine added to the media. The addition of TDZ to the media for the chilled *T. kaufmanniana* embryo culture resulted in a higher regeneration of bulblets compared to the addition of BAP in the same concentration.

In vitro germination of *T. kaufmanniana* embryos provides an efficient and alternative way of breaking the dormancy in this threatened plant species by exposing the explants to different chilling or warm temperatures for certain periods of time.

Author Contribution

N.A.Y[0000-0001-5075-6007]**:** designed the in vitro experiments, performed statistical analysis and edited the manuscript; **G.L.V**[0000-0003-1884-1206]**:** studied the ontogeny of the plants in the introduction collection, evaluated the efficiency of the generative reproduction of the species and wrote the first draft of the manuscript. Both authors read and approved the final manuscript.

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