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Practical Methods for Breaking Seed Dormancy in a Wild Ornamental Tulip Species *Tulipa thianschanica* Regel

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Abstract: The innate physiological dormancy of *Tulipa thianschanica* seeds ensures its survival and regeneration in the natural environment. However, the low percentage of germination restricts the establishment of its population and commercial breeding. To develop effective ways to break dormancy and improve germination, some important factors of seed germination of *T. thianschanica* were tested, including temperature, gibberellin (GA_3) and/or kinetin (KT), cold stratification and sowing depth. The percentage of germination was as high as 80.7% at a constant temperature of 4 °C, followed by 55.6% at a fluctuating temperature of 4/16 °C, and almost no seeds germinated at 16 °C, 20 °C and 16/20 °C. Treatment with exogenous GA_3 significantly improved the germination of seeds, but KT had a slight effect on the germination of *T. thianschanica* seeds. The combined treatment of GA_3 and KT was more effective at enhancing seed germination than any individual treatment, and the optimal hormone concentration for the germination of *T. thianschanica* seeds was 100 mg/L GA_3 + 10 mg/L KT. In addition, it took at least 20 days of cold stratification to break the seed dormancy of *T. thianschanica*. The emergence of *T. thianschanica* seedlings was the highest with 82.4% at a sowing depth of 1.5 cm, and it decreased significantly at a depth of >3.0 cm. This study provides information on methods to break dormancy and promote the germination of *T. thianschanica* seeds.

Keywords: dormancy; hormone; stratification; seed; temperature; *Tulipa thianschanica*

1. Introduction

Tulipa thianschanica Regel, a wild species of tulip in the Liliaceae, grows on hills and steppes at an altitude from 1000 m to 1800 m in western Xinjiang, China and the Central Asian regions [1]. It is known for its unusual shape and brightly colored flowers. Currently, the population of *T. thianschanica* is quickly declining owing to unreasonable exploitation and destructive grazing in its native area. *T. thianschanica* is propagated in situ primarily through seeds, but seed dormancy results in low percentage of germination [2]. Thus, effective measures should be taken to maintain its survival, enhance its propagation and make use of it in landscapes.

Dormancy is a biological adaptation of seeds that is regulated by many factors. Temperature is a major environmental factor responsible for various changes in seed dormancy, and dormancy can be broken by fluctuating temperatures or warm/cold stratification. In general, fluctuating temperatures have been reported to promote seed germination more than constant temperatures [3,4]. However, for tulips,

as reported by Tang, the seeds of *T. iliensis* required a period of cold stratification to germinate [5]. Rouhi also found that the seed dormancy of *T. kaufmanniana* could be broken by cold stratification for 49 days [6]. A treatment of constant cold had been proven to be more effective than fluctuating temperatures in tulips [7]. Although a substantial amount of research on the seed dormancy of tulip has been conducted, the definite temperature for its release from dormancy is unknown, and the response of *T. thianschanica* seeds to temperature has not yet been reported.

Both abscisic acid (ABA) and gibberellin (GA) play an important role in the regulation of dormancy, and dormancy is controlled by a balance between ABA and GA [8]. The synthesis of ABA at radical blocks embryo growth, and the maintenance of dormancy requires the biosynthesis of ABA [9]. The presence of ABA in the endosperm of *Arabidopsis* seed was a key component that inhibited the growth of embryos [10]. GA is a positive regulator for seed germination, and the application of exogenous GA affected the level of seed dormancy of *T. iliensis* and *T. tarbagataica* [11]. Cytokinins (KT) are known to be involved in cell differentiation and are essential for plant growth [12]. In particular, KT alone was able to break seed dormancy [13]. Lee also reported that KT improved the dehiscence of seed and their germination in ginseng [14]. There is no doubt that the regulation of hormones is involved in seed dormancy and germination, while the manner in which they worked in *T. thianschanica* has not been determined.

The habit or type of seed dormancy determines the ecological niche of its germination and propagation, which is substantially related to factors of climate, humidity, soil, light, nutrients and biological and abiotic stresses [15,16]. Stratification and burial depth are also considered to be the key factors for seed germination in many plants. For example, Nie determined that stratification at fluctuating temperatures (2/15 °C) was effective for the release of seed dormancy in *T. iliensis* [17]. A study showed that the seed dormancy of *T. sinkiangensis* could be broken only by 8 weeks of cold stratification at 4 °C [18]. Zhang also came to a similar conclusion for *T. iliensis* [19]. Previously, Qu reported a sowing method for tulip seeds and concluded that the most suitable sowing depth was 1.5–2.5 cm [20]. The duration of stratification and the sowing depth affected the percentage of seed germination and seedling emergence, but there has been no report on these factors in *T. thianschanica*.

Increasing the percentage and speed of germination of *T. thianschanica* had a positive economic effect for nurseries by increasing the production of seedlings within a short period. Additionally, it would be a valuable technical strategy for germplasm resources to use in the conservation of wild tulips. In this study, we investigated the effects of temperature, hormone, cold stratification and sowing depth on seed germination in *T. thianschanica* and focused on revealing their interactions, which would be significant to further explore the mechanism of seed dormancy.

2. Materials and Methods

2.1. Seed Collection

Nearly dehiscent capsules of *T. thianschanica* were harvested from the National Tulip Germplasm Repertory of China, Liaoning Academy of Agriculture Sciences, Shenyang, China (Longitude: 123°55′ E; Latitude: 40°03′ N) in 2016. Seeds that contained embryos were selected for the germination test. All freshly dried seeds with water content <10% [21] were collected in June 2016 and stored in a naturally ventilated room for approximately one month until use.

The seeds were sterilized by 75% ethanol for 3 min, rinsed three times with distilled water and then dried on a clean bench. The 100 seeds in each treatment were placed in an 11 cm diameter Petri dish with two layers of filter paper, and 5 mL of distilled water were added. The filter papers were kept moist by the addition of distilled water during seed culture. Three replicates were used for each treatment. The seeds were considered to be germinating when the cotyledonary petiole emerged and were at least 1–2 mm long.

The date of initial germination was considered to be when 5% of the seeds had germinated. Seed germination was recorded daily until no further seeds were germinating in any replication for 15 continuous days, which was defined as the date of final germination. The germination (%) was calculated for each treatment by the following formula:

$$\text{Germination percentage} = \text{No. of seeds germinated/No. of total seeds} \times 100\% \quad (1)$$

The speed of germination that was expressed as the germination index (*GI*) was calculated by the following formula as described by Hu et al. (2012) [22]:

$$GI = \sum(\text{Gt/Dt}) \quad (2)$$

where Gt is the number of seeds germinated on date t and Dt is the day number of the corresponding date.

The time of 50% seed germination (T_{50}) was calculated by the following formula, as described by Huang et al. (2017) [23]:

$$T_{50} = \text{di} + ((\text{N}/2 - \text{ni}) \times (\text{dj} - \text{di}))/(\text{nj} - \text{ni}) \quad (3)$$

where N is the final number of seeds that germinated and ni and nj (ni < N/2 < nj) are the total numbers of seed germinated by adjacent counts at di and dj times.

2.2. Water Imbibition and Seed Coat Removal Tests

Seeds on moist filter paper in Petri dishes were incubated at 4 °C. The fresh weight of seeds was measured hourly on an electronic scale until it remained steady. The water content increment (WC) and water absorption rate (WR) were calculated using the following formulas as described by Nijenstein et al. (2002) [21]:

$$\text{WC} = (\text{Mt} - \text{M0})/\text{Mt} \times 100\% \quad (4)$$

$$\text{WR} = (\text{WCt} - \text{WC0})/(\text{WCmax} - \text{WC0}) \times 100\% \quad (5)$$

where Mt is the seed weight after water absorption at t time, M0 is the initial seed weight, WCt is the percentage of seed water content at t time, WC0 is the percentage of initial water content and WCmax is the percentage of maximum seed water absorption.

To detect the interaction between seed coat and seed dormancy, two treatments were conducted with three replicates. In the first treatment, 100 intact seeds were directly incubated at 4 °C and 4/16 °C. In the second treatment, the coats of 100 seeds were removed by forceps on a clean bench after 3 h of imbibing and were then incubated at a constant temperature of 4 °C and a fluctuating temperature of 4/16 °C.

2.3. Temperature Test

The seeds were cultured in an incubator with constant temperatures and fluctuating temperatures in the dark. The constant temperatures were 4, 8, 12, 16 and 20 °C. The four types of fluctuating temperature treatments were conducted as follows: (A) four fluctuating temperatures with a 4 °C interval (4/8 °C, 8/12 °C, 12/16 °C, 16/20 °C), (B) two fluctuating temperatures with an 8 °C interval (4/12 °C, 12/20 °C), (C) one fluctuating temperature with a 12 °C interval (4/16 °C) and (D) one fluctuating temperature with a 16 °C interval (4/20 °C).

2.4. Plant Growth Regulators Test

We tested the effects of three plant growth regulators (PGR) on germination on *T. thianschanica* seeds. According to Rouhi [6], three concentrations of each PGR were conducted. We tested 50, 100 and 200 mg/L gibberellin (GA_3); 5, 10 and 20 mg/L kinetin (KT); and 1.0, 2.0 and 4.0 mg/L potassium nitrate (KNO_3). Concentration combinations of 3 × 3 were conducted to determine the interaction of two hormones on the seed germination. In each treatment, 100 seeds were incubated at 4 °C in Petri dishes on two layers of filter paper moistened with 5 mL of solution. Water without PGR was used as

the control. Each treatment was conducted in triplicate. All the plant growth regulators used in this experiment were purchased from Sigma-Aldrich Trade Co., Ltd. (Shanghai, China).

2.5. Stratification Test

Seeds were placed in Petri dishes on two layers of filter paper saturated with 5 mL distilled water or GA_3 (100 mg/L), KT (20 mg/L), KNO_3 (2.0 mg/L) or GA_3 (100 mg/L) + KT (10 mg/L). Seeds were then stratified in the dark for 15, 20 and 25 days at a constant temperature 4 °C. After cold stratification, all seeds were transferred to a greenhouse (<20 °C) for germination under natural light.

2.6. Seed Sowing Depth Test

Seeds were sown at four depths (0, 1.5, 3.0 and 4.5 cm) in a sowing box filled with substrate that was purchased from the Jiffy Company in 2016. The substrate depth in the sowing box was 13–15 cm after compaction. Three replicates were used for each sowing depth and each box was sown with approximately 1000 seeds. After sowing, the surface was covered with 0.3 cm thick pure sand, fully moistened gently, and the sowing boxes were transferred to a cold storage room for stratification. After one month of stratification at 4 °C, the sowing boxes were placed in a greenhouse that was approximately 15–20 °C during the day and watered by spraying. A seedling was considered to have emerged when its needle-shaped cotyledon was visible. The number of seedlings that emerged was recorded daily for 40 days. Droppers (small bulbs) were harvested in July of 2017 and 2018 and were planted at different depths in December of the year that they were harvested. The survival of seedlings each year was counted.

2.7. Statistical Analysis

The data was analyzed for significance using a one-way analysis of variance (ANOVA) in the program SPSS v. 19.0 (IBM, Inc., Armonk, NY, USA). The mean data were compared with Duncan's multiple range test to determine significant differences ($p < 0.05$). All the figures were produced using Origin 8.0 (OriginLab, Northampton, MA, USA).

3. Results

3.1. Water Absorption and Testa Influence on Seed Germination

T. thianschanica seeds absorbed water very quickly. When the seeds were incubated for 4 h, the WR reached 82.0%, and the WC reached 51.2%. The change in WR was <8.0% over the imbibition time from 4 to 6 h, and the WC and WR almost reached their peak values at 6 h.

There was no significant difference in germination between the intact and coatless seeds when they were treated at a constant 4 °C. Both treatments reached 85.0% after 60 days. When the seeds were treated at a fluctuating 4/16 °C, the germination percentage of the coatless seeds was significantly higher than that of the intact seeds, whereas neither of them exceeded 20.0% (Figure 1A). The germination index (*GI*) of the coatless seeds (5.4) was significantly higher than that of the intact seeds (4.7) at a constant 4 °C, while there was no significant difference at a fluctuating 4/16 °C (Figure 1B). In brief, the seed coat did not affect its germination.

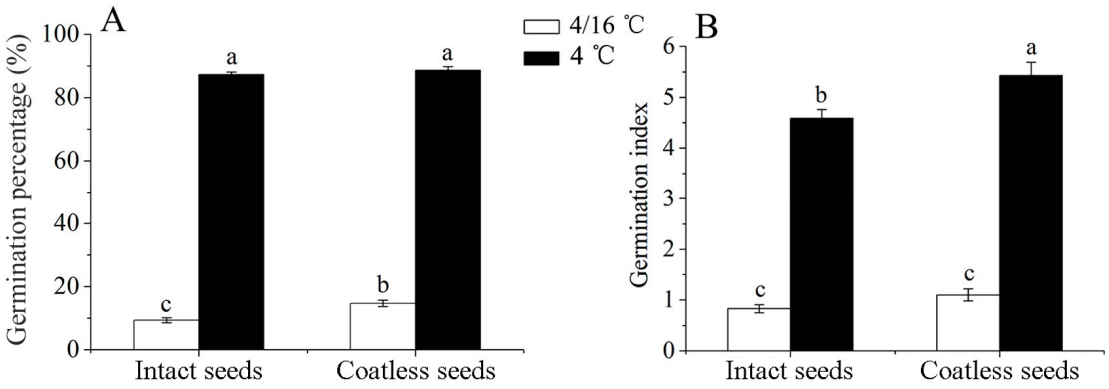

Figure 1. The germination percentage (**A**) and germination index (**B**) of intact seeds and coatless seeds of *Tulipa thianschanica* at a constant temperature of 4 °C and a fluctuating temperature of 4/16 °C. The different lowercase letters indicate a significant difference at $p < 0.05$ with Duncan's multiple range test.

3.2. Effect of Temperature on Seed Germination

As shown in Figure 2, with the increase in temperature, the percentage of seed germination of *T. thianschanica* decreased significantly. The highest germination (80.7%) was observed at a constant temperature of 4 °C, and no seeds germinated at a temperature higher than 16 °C. The increasing interval between the two temperatures positively affected germination, which increased significantly from 18.3% (4/8 °C) to 55.6% (4/16 °C). However, the seed germination was less than 5.0% at a fluctuating temperature of 12/16 °C, and no seeds germinating at 16/20 °C and 12/20 °C.

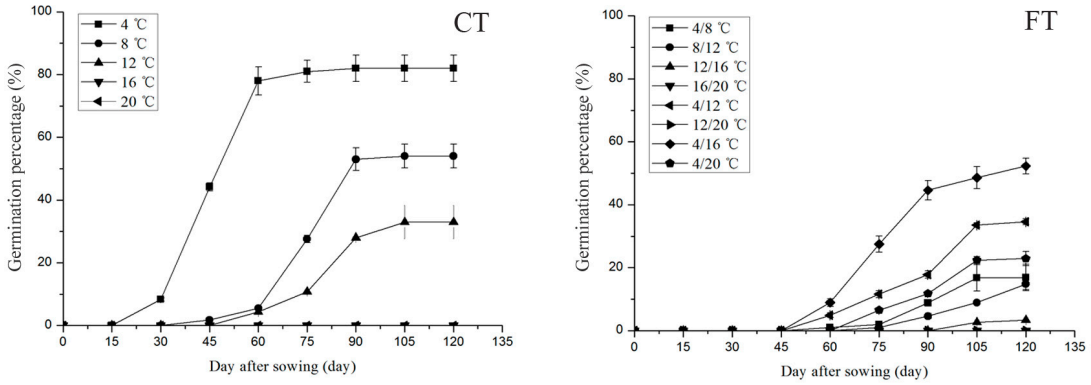

Figure 2. Effects of constant temperatures (CT) and fluctuating temperatures (FT) on the seed germination of *Tulipa thianschanica.* Different lowercase letters indicate a significant difference at $p < 0.05$ with Duncan's multiple range test.

The days on which *T. thianschanica* seeds germinated differed substantially among the various treatments (Table 1). The seeds started to germinate at about 30 days when they were treated at 4 °C, followed by about 60 days when seeds were treated at 8 °C and 4/16 °C. The lowest T_{50} seeds was 46.1 days at 4 °C and 78.3 days at 4/16 °C. The T_{50} of the remaining temperature treatments exceeded 90 days (Table 1). It took only 8 weeks for seeds to complete germination at 4 °C, but it took twice as long at 4/16 °C. In summary, the optimal temperature for the germination of *T. thianschanica* seeds was 4 °C.

Table 1. Germination days of *Tulipa thianschanica* seeds at different temperatures.

Temperatures (°C)	Initial Germination Duration (Day)	T_{50} (Day)	Final Germination Duration (Day)
	CT[x]		
4	33.8 ± 1.08 [d]	46.1 ± 1.35 [d]	57.3 ± 1.66 [c]
8	61.3 ± 2.20 [bc]	89.2 ± 2.11 [b]	107.3 ± 2.45 [b]
12	63.5 ± 2.35 [b]	92.2 ± 2.01 [b]	111.0 ± 1.88 [b]
16	–[z]	–	–
20	–	–	–
	FT[y]		
4/8	69.3 ± 3.32 [a]	92.3 ± 2.89 [b]	118.3 ± 4.05 [ab]
8/12	72.0± 1.88 [a]	97.2 ± 1.28 [a]	128.6 ± 2.19 [a]
12/16	73.3 ± 4.41 [a]	95.9 ± 0.88 [a]	126.0 ± 5.34 [a]
16/20	–	–	–
4/12	64.0± 6.24 [b]	97.4 ± 2.33 [a]	121.6 ± 3.71 [a]
12/20	–	–	–
4/16	59.2 ± 4.81 [c]	78.3 ± 4.72 [c]	102.3 ± 3.76 [b]
4/20	72.6 ± 2.60 [a]	91.5 ± 2.64 [b]	126.6 ± 3.46 [a]

CT[x] Constant temperature treatments. FT[y] Fluctuating temperature treatments. [z] – represent no germination observed. The different lowercase letters within each column show a significant difference at $p < 0.05$ with Duncan's multiple range test.

3.3. Effect of Exogenous Plant Growth Regulators on Seed Germination

To improve germination, three plant growth regulators (PGR) were used in addition to cold stratification at 4 °C. The percentage of seed germination was significantly affected by PGR. GA_3 significantly improved the germination of *T. thianschanica* seeds to 83.7%, regardless of the concentration (Figure 3A). In addition, KT (20 mg/L) also improved the seed germination to 82.7% compared with the non-treated seeds (Figure 3B). Compared with the control, a low concentration of KNO_3 had a positive effect on seed germination, but it did not improve the total germination (75.6%) of *T. thianschanica* seeds (Figure 3C).

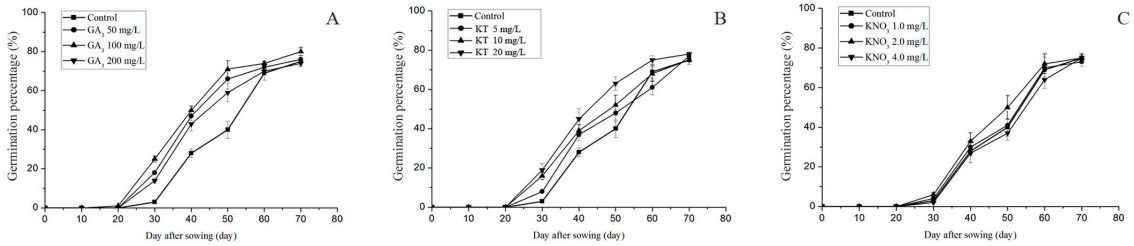

Figure 3. Effect of plant growth regulators on the seed germination of *Tulipa thianschanica*. (**A**) GA_3 (Gibberellin); (**B**) KT (Kinetin); (**C**) KNO_3.

As shown in Table 2, the initial germination day of *T. thianschanica* seeds using GA_3 and KT alone was 26.9–32.6 days and 28.2–30.0 days, respectively. The T_{50} was significantly shorter when seeds were treated with GA_3 (41.3–44.6 days) and KT (46.6–47.3 days) than in the control (57.4 days), regardless of their concentrations. Compared with the control, treatment with GA_3 significantly promoted seed germination 1–2 weeks ahead of the control, and the KT treatment also showed a positive effect on seed germination. In our study, KNO_3 also had a positive effect on germination, but germination duration was insignificant compared with that of the control.

To detect the interaction of two hormones on seed germination, nine treatments were conducted. KT alone had little effect on germination, but a mixture of 100 mg/L GA_3 and 10 mg/L KT significantly shortened the duration of initial germination from 36.2 days to 25.3 days, T_{50} from 55.3 days to 42.3 days, and the final germination duration from 69.5 days to 49.8 days (Table 3). The combination of GA_3 and KT was more effective for promoting germination than using either hormone alone. In other words, GA_3 and KT had a positive effect on seed germination, and the best combination was GA_3 100 mg/L + KT 10 mg/L.

Table 2. The seed germination days of *Tulipa thianschanica* incubated with different plant growth regulator treatments at 4 °C.

Treatments (mg/L)		Initial Germination Duration (Day)	T_{50} (Day)	Final Germination Duration (Day)
Control	0	35.7 ± 1.45 ^{a}	57.4 ± 1.94 ^{a}	65.8 ± 3.76 ^{a}
GA_3	50	28.6 ± 1.24 ^{cd}	42.7 ± 1.20 ^{c}	53.3 ± 1.33 ^{c}
	100	26.9 ± 1.85 ^{d}	41.3 ± 0.82 ^{c}	52.6 ± 0.39 ^{c}
	200	32.6 ± 1.53 ^{b}	44.6 ± 1.20 ^{bc}	57.3 ± 1.21 ^{bc}
KT	5	30.0 ± 1.52 ^{c}	47.0 ± 1.63 ^{b}	63.3 ± 0.89 ^{ab}
	10	28.5 ± 1.26 ^{cd}	47.3 ± 1.45 ^{b}	62.0 ± 0.58 ^{ab}
	20	28.2 ± 1.18 ^{cd}	46.6 ± 1.20 ^{b}	60.7 ± 0.85 ^{b}
KNO_3	1.0	32.6 ± 1.45 ^{b}	56.0 ± 1.15 ^{a}	64.0 ± 2.08 ^{ab}
	2.0	31.0 ± 1.53 ^{bc}	55.2 ± 2.59 ^{a}	63.2 ± 3.21 ^{ab}
	4.0	35.6 ± 2.72 ^{a}	56.5 ± 2.34 ^{a}	63.6 ± 8.37 ^{ab}

The different lowercase letters within each column show significant differences at $p < 0.05$ with Duncan's multiple range test. GA_3, gibberellin; KT, kinetin.

Table 3. Effect of the combination of GA_3 and KT on the seed germination of *Tulipa thianschanica*.

PGRs (mg/L)		Initial Germination Duration (Day)	T_{50} (Day)	Final Germination Duration (Day)
GA_3	KT			
0	0	36.2 ± 0.32 ^{a}	55.3 ± 0.66 ^{a}	69.5 ± 2.01 ^{a}
50	5	29.3 ± 1.86 ^{c}	43.0 ± 1.34 ^{c}	64.0 ± 2.65 ^{b}
	10	31.4 ± 2.16 ^{b}	44.3 ± 2.10 ^{c}	55.6 ± 1.44 ^{c}
	20	27.5 ± 1.44 ^{cd}	42.6 ± 1.02 ^{cd}	53.1 ± 0.96 ^{d}
100	5	27.8 ± 1.96 ^{cd}	46.5 ± 1.22 ^{b}	54.2 ± 1.32 ^{cd}
	10	25.3 ± 2.53 ^{d}	42.3 ± 2.10 ^{d}	49.8 ± 0.53 ^{e}
	20	25.5 ± 1.19 ^{d}	43.1 ± 1.34 ^{c}	54.4 ± 1.34 ^{cd}
200	5	29.4 ± 2.64 ^{c}	46.6 ± 2.36 ^{b}	55. 5 ± 3.28 ^{c}
	10	26.0 ± 0.49 ^{d}	43.2 ± 1.74 ^{c}	52.6 ± 1.37 ^{d}
	20	32.3 ± 3.02 ^{b}	44.3 ± 2.67 ^{c}	56.7 ± 2.58 ^{c}
Significance				
GA_3		***	**	**
KT		**	*	ns
$GA_3 \times KT$		***	***	***

The different lowercase letters within each column show significant differences at $p < 0.05$ with Duncan's multiple range test. ns, non-significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. GA_3, gibberellin; KT, kinetin; PGRs, plant growth regulator.

3.4. Effect of Cold Stratification on Seed Germination

Cold stratification was an effective way of breaking the seed dormancy of *T. thianschanica*. Few seeds germinated when stratified for 15 days at 4 °C regardless of any other treatment (Figure 4). After 20 days of cold stratification at 4 °C, only <27.5% seeds germinated in the control and 38.6%, 34.2% and 31.2% seeds germinated with treatments of GA_3, KT and KNO_3 alone, respectively. The highest seed germination was recorded in GA_3 + KT (47.5%). There was 50.0% germination after 25 days, but >70.0% of the seeds germinated when exogenous hormone was added, i.e., germination increased by nearly 1.5 times of the control (52.6%).

The stratification duration had a significant effect on the time of germination (Table 4). There was no seed germination after cold stratification for 15 days among the control treatments, KT and KNO_3, and few germinated seeds were observed in the treatments with GA_3 and GA_3 + KT. Germination was insignificant in the initial germination time, T_{50} and final germination time after cold stratification for 15 days. Similar to the former, both the KT and KNO_3 treatments differed insignificantly compared with the control in initial germination time, T_{50}, and the final germination time after cold stratification for 20 days, whereas the seeds germinated 6 to 8 days earlier when they were treated by GA_3 and GA_3 + KT (Table 4), and the initial germination time was significant ($p < 0.05$). T_{50} and the final germination time was significant ($p < 0.01$). After 25 days of cold stratification, the PGR treatments significantly shortened the germination time (Table 4), and the initial germination time, T_{50} and the final germination time were improved significantly ($p < 0.001$). The shortest initial germination time

(27.5 days) was observed in the GA_3 + KT treatments after 25 days of cold stratification, and the seed germination was completed one month earlier than in the control. The most effective stratification duration for the seed germination was 25 days considering the germination percentage and time.

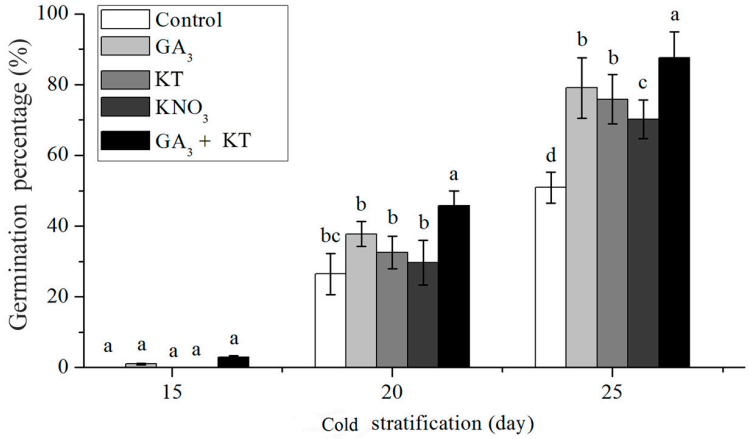

Figure 4. Effect of cold stratification combined with plant growth regulator treatments on the seed germination of *Tulipa thianschanica*. The different lowercase letters indicate a significant difference at $p < 0.05$ with Duncan's multiple range test.

Table 4. Effect of the duration of stratification on *Tulipa thianschanica* seed germination at 4 °C with optimal concentrations of plant growth regulators.

Cold Stratification Duration (Day)	Treatments (mg/L)	Initial Germination Duration (Day)	T_{50} (Day)	Final Germination Duration (Day)
15	Control (0)	– [z]	–	–
	GA_3 (100)	74.0 ± 3.60 [a]	93.0 ± 2.65 [a]	133.7 ± 3.18 [a]
	KT (20)	–	–	–
	KNO_3 (2.0)	–	–	–
	GA_3 (100) + KT (10)	73.6 ± 4.07 [a]	91.6 ± 5.02 [a]	132.3 ± 5.24 [a]
	Significance	ns	ns	ns
20	Control (0)	59.7 ± 2.19 [a]	76.7 ± 3.93 [a]	97.6 ± 4.91 [a]
	GA_3 (100)	53.8 ± 2.08 [b]	70.2 ± 2.08 [b]	85.7 ± 7.80 [b]
	KT (20)	57.3 ± 2.73 [a]	74.5 ± 3.76 [a]	93.0 ± 5.20 [a]
	KNO_3 (2.0)	57.2 ± 2.19 [a]	75.7 ± 2.81 [a]	95.3 ± 1.35 [a]
	GA_3 (100) + KT (10)	51.4 ± 3.46 [b]	64.9 ± 2.18 [c]	80.3 ± 2.08 [c]
	Significance	*	**	**
25	Control (0)	43.5 ± 1.53 [a]	61.3 ± 4.04 [a]	77.5 ± 3.69 [a]
	GA_3 (100)	31.3 ± 0.89 [c]	48.3 ± 1.76 [c]	56.5 ± 1.20 [c]
	KT (20)	36.2 ± 3.06 [b]	53.7 ± 3.84 [b]	67.5 ± 2.31 [b]
	KNO_3 (2.0)	38.0 ± 1.15 [b]	54.3 ± 2.03 [b]	65.0 ± 2.32 [b]
	GA_3 (100) + KT (10)	27.5 ± 2.08 [d]	43.6 ± 3.18 [d]	50.7 ± 1.20 [d]
	Significance	***	***	***

[z] – indicates that no germination was observed. The different lowercase letters within each column show significant differences at $p < 0.05$ with Duncan's multiple range test. Statistical significance determined within the same stratification days: ns, non-significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. GA_3, gibberellin; KT, kinetin.

3.5. Effect of Seed Sowing Depth on Seedling Emergence

The seedling emergence and growth were observed from 2017 to 2019. In 2017, the seedling emergence (approximately 80.0%) was not significant among the 0 cm, 1.5 cm and 3.0 cm sowing depths, while all of them were significantly higher than that at the 4.5 cm sowing depth. In 2018, compared with a depth of 1.5 cm (71.3%), the seedling emergence rate decreased significantly to 60.2% at 0 cm, 57.7% at 3.0 cm and 32. 5% at 4.5 cm. In 2019, the same trend was also observed.

The highest seedling emergence percentage occurred at a sowing depth of 1.5 cm, and the lowest seedling emergence percentage occurred at a sowing depth of 4.5 cm. As a result, the sowing depth significantly affected the seedling emergence of *T. thianschanica*, and the optimal sowing depth was 1.5 cm.

4. Discussion

4.1. Temperature Regulation on Plant Seed Germination

Many researchers have shown that the seeds of tulip species require a long period of low temperature and substantial humidity for germination [24]. Nabieva and Gerasimovich reported that the formation of adventitious bulbs was zero when the isolated *T. kaufmanniana* embryos were non-chilled, which confirmed that low temperature (4 °C) played an important role in seed germination [25]. According to Yuan and Mu, the mean temperature of early spring (January to March) was 4 °C in the Tianshan Mountains of Xinjiang, China, and the maximum daytime temperature approached nearly 16 °C [26]. In our experiments, the highest germination percentage of *T. thianschanica* seed was observed at a constant temperature of 4 °C and/or a fluctuating temperature regime of 4/16 °C. Our studies demonstrated that the seeds were responsive to treatments at different temperatures. Few or no seeds germinated at 16 °C and 16/20 °C, which indicated that 16 °C might serve as a threshold value for the germination of *T. thianschanica* seed. That is, high temperatures prevented dormancy break and/or germination.

4.2. Plant Growth Regulators Regulation on Plant Seed Germination

Phytohormones were involved in seed germination, particularly gibberellin (GA) and abscisic acid (ABA) [27–29], and it was confirmed that the combination of them had antagonistic effects on seed germination [30,31]. Gibberellin, which releases the dormancy of seeds, played a key role in promoting the rupture of testa [32,33]. Aghilian reported that GA_3 significantly increased the seed germination percentage of *Plantago ovate*, *Rudbeckia hirta* and *Satureja hortensis* [34]. Similarly, GA_3 also increased the speed of germination in the seeds of *T. iliensis* and *T. sinkiangensis* [35]. Our results indicated that 50 mg/L or 100 mg/L GA_3 significantly increased the germination percentage of *T. thianschanica* seeds. However, it was worth noting that when the concentration of GA_3 increased to 200 mg/L, the effect on the seed germination of *T. thianschanica* became negative. Ye found a similar result that GA_3 (100 μM) could break the dormancy of sunflower and Egyptian broomrape, but it produced a negative effect on seed germination when the concentration of GA_3 was increased further [36]. Our results showed that the combination treatments of GA_3 and KT had a synergistic effect on the seed germination of *T. thianschanica*, which was consistent with those of Kelly and Lacroix, who reported that the combination of GA_3 and KT improved the germination of *Symphyotrichum* seeds [37]. Similarly, the combination of GA_3 and KNO_3 also increased the germination time of *T. scardica* and *T. kosovarica* [38]. To our knowledge, this was the first report of a synergistic effect of GA_3 and KT on the seed germination of *T. thianschanica*, and the optimal hormone combination for seed germination was GA_3 100 mg/L + KT 10 mg/L.

Nitrogenous compounds, such as nitrate, NO and cyanide, have a positive effect on the release of dormancy [39–41]. Wang reported that the application of nitrate in potato promoted the expression of a gene involved in ABA catabolism (*StCYP707A1*) [42]. Nitrate acted as a signal regulating substance rather than a nutrient that reduced the level of dormancy. During seed development and germination, nitrate induced the transcription of *CYP707A2* gene and then affected seed dormancy [43,44]. In our study, KNO_3 also promoted seed germination to some extent, although it was not significant.

4.3. Cold Stratification Regulation on Plant Seed Germination

Cold stratification was an effective way to alleviate seed dormancy [45]. It played a key role in the release of seed dormancy of *T. thianschanica* in our experiment. Rouhi concluded that stratification for 49 days was more effective than that for 35 days in *T. kaufmanniana* Regel [46]. Moreover, our study

confirmed that seeds that had been stratified at 4 °C for less than 15 days could only partially germinate, while almost all the seeds could germinate after cold stratification for 25 days. Similarly, Tang reported that the seeds needed a period of more than 4 weeks of cold stratification to break dormancy in *T. iliensis* and *T. sinkiangensi* [5]. The F_1 seeds of an interspecific hybrid between 'Negrita' and *T. thianschanica* also required 36–67 days of cold stratification (4 °C) to germinate [47]. These results implied that the seeds of *T. thianschanica* survived in the soil for a long period in the hot summer and germinated in early spring when the temperatures were favorable. Our study provides clear evidence that the responses of seed germination to temperature and hormones depend on the duration of stratification in *T. thianschanica*.

5. Conclusions

In summary, seed dormancy of *T. thianschanica* was not imposed by its testa. A constant temperature of 4 °C and a fluctuating temperature of 4/16 °C were the most suitable promoting germination of *T. thianschanica*. Both GA_3 and KT treatments improved the germination of *T. thianschanica*. GA_3 significantly shortened the germination time, and KT also had a positive effect on seed germination. The combination treatments of GA_3 and KT were more effective than those used alone to promote seed germination. Cold stratification for more than 25 days was the key step for the germination of *T. thianschanica* seed. GA_3 and/or KT treatments could completely substitute for the cold stratification requirement to break dormancy and promote germination in *T. thianschanica* seeds. The sowing depth affected seedling emergence of *T. thianschanica*, and the most suitable depth was 1.5 cm. Our research has found effective practical methods to promote the seed germination of an important wild ornamental tulip species.

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