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Phylogenetic trends in TZ staining analysis of six deep dormancy seeds

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Abstract

The assessment of seed quality and physiological potential is essential in seed production and crop breeding. In the process of rapid detection of seed viability using tetrazolium (TZ) staining, it is necessary to spend a lot of labour and material resources to explore the pretreatment and staining methods of hard and solid seeds with physical barriers. This study explores the TZ staining methods of six hard seeds (*Tilia miqueliana*, *Tilia henryana, Sassafras tzumu*, *Prunus subhirtella*, *Prunus sibirica*, and *Juglans mandshurica*) and summarizes the TZ staining conditions required for hard seeds by combining the difference in fat content between seeds and the kinship between species, thus providing a rapid viability test method for the protection of germplasm resources of endangered plants and the optimization of seed bank construction. The TZ staining of six species of hard seeds requires a staining temperature above 35 °C and a TZ solution concentration higher than 1%. Endospermic seeds require shorter staining times than exalbuminous seeds. The higher the fat content of the seeds, the lower the required incubation temperature and TZ concentration for staining, and the longer the staining time. And the closer the relationship between the two species, the more similar their staining conditions become. The TZ staining method of similar species can be predicted according to the genetic distance between the phylogenetic trees, and the viability of new species can be detected quickly.

Introduction

Seed viability refers to the potential germination capacity of a seed or the vitality of the seed embryo (Finch-Savage and Leubner-Metzger, 2006; Rajjou et al., 2012). The assessment of seed quality and physiological potential is essential in seed production and crop breeding (Matthews et al., 2012). The seed industry needs sound information about the viability of seed lots within a short time to make quick decisions on seed marketing (Finch-Savage and Bassel, 2016). Seed quality includes physical quality, health status and physiological quality (Gaur et al., 2020). Among the various methods of seed viability testing, germination testing is undoubtedly the most accurate assessment, but germination testing requires more time and a lot of labour and material resources, and some seeds require special pretreatment methods; for example, dormant seeds need to break dormancy before germination (Copeland et al., 2001). Methods such as near-infrared reflectance spectroscopy, hyperspectral imaging (HSI) and X-ray scanning can detect seed viability non-destructively and quickly, but they are not very accurate and have large errors, making it difficult to obtain accurate viability levels for seeds from different batches and species (Ambrose et al., 2016; Al-Turki and Baskin, 2017; Pang et al., 2021). The use of reactive dyes to determine the viability of different seeds is currently the best alternative to germination tests for rapid and accurate viability determination (Pritchard, 1985). Among these, the tetrazolium (TZ) test is one of the most commonly used seed viability tests to date and has the advantage of being a rapid test (Magrini et al., 2019). This is particularly useful for freshly harvested seeds that have high levels of dormancy and are difficult to germinate such as some grasses, trees and crops (Conn et al., 2006; Souza et al., 2010; França-Neto and Krzyzanowski, 2022). The results of the TZ test indicate the number of viable seeds in a sample that are capable of producing normal plants under suitable

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germination conditions. While a germination test takes 3–4 weeks to complete for most grass species, a TZ test can be complete within 24–48 h (Elias et al., 2006).

Employing TZ testing presents a challenge due to the need for taxon-specific seed pretreatment and staining methods. This includes TZ concentration, incubation time and incubation temperature, which often require significant human and material resources to explore. It is worth noting that closely related species often have similar morphological, ecological and physiological characteristics (Clausen et al., 1941; Burns and Strauss, 2011), such as fat content, which is closely related to TZ-staining conditions (Thom et al., 1993). If possible, predicting optimal testing conditions based on phylogenetic proximity could help identify the best TZ-staining method for seeds of new species without the need for experimentation. This would allow for quick viability measurements and save labour and material resources.

To investigate the relationship between phylogenetic relatedness, seed traits and optimal TZ testing conditions, we identified a group of hard-coated species with deep dormancy. The seeds of six species (*Tilia miqueliana*, *Tilia henryana*, *Sassafras tzumu*, *Prunus subhirtella*, *Prunus sibirica*, and *Juglans mandshurica*) were all deeply dormant and hard-coated. Hard seeds refer to the impermeable barrier cell layer covered with a complete cuticle in the outer seed coat or shell (Finkelstein et al., 2008). The cell layer inhibits seed germination by blocking water and oxygen from the external environment, rendering the seed physically dormant (Finch-Savage and Leubner-Metzger, 2006; Steinbrecher and Leubner-Metzger, 2017). Only after the outer cuticle layer has been weakened will the seed absorb water and germinate (Egley, 1989). Common treatments include acid etching, hot water soaking, mechanical damage, high pressure and variable temperature treatments (Rifna et al., 2019; Dai et al., 2023). These treatments are all time-consuming and laborious, making quality seed inspections difficult (Rosbakh et al., 2019).

T. miqueliana and *T. henryana* are rare species of the genus *Tilia* of the Malvaceae, *S. tzumu* is a deciduous tree of the Lauraceae, and they are found only in some provinces of China and are rare in number (Xiao and Zhou, 1988; Shi et al., 2012). The seeds have the characteristics of deep dormancy, which can partially germinate after 2–3 years in the natural state (Qiang et al., 2007). After the dormancy-breaking treatment, the dormancy can be broken after 2–8 months of low temperature stratification (Wu et al., 2021; Chen et al., 2022; Peng et al., 2023). *P. subhirtella* and *P. sibirica* belong to the genus *Prunus* of the Rosaceae, have relatively shallow dormancy characteristics and still require H_2SO_4 corrosion, gibberellin (GA) immersion and stratification to break the dormancy (Wang et al., 2008; Khudonogova et al., 2019). *J. mandshurica* is an arboreal plant belonging to the genus *Juglans* in the Juglandaceae. Its seeds have hard shell, dense structure and deep physical dormancy and are difficult to germinate (Jin, 2022). In the process of rapid testing of viability by TZ staining, a lot of labour and material resources will be spent on exploring the seed pretreatment and staining methods. Therefore, the general rules for staining seeds of different types and characteristics (especially hard seeds) derived from distance and genetic evolutionary trees are of great value in reducing the exploration time and cost, and provide references for the protection of germplasm resources of endangered plants and the optimization of seed bank operations.

Materials and methods

Plant material

All seeds were collected from cultivated plants. *T. henryana* seeds were collected from Yangshu Forest Farm (119°10′ E, 31°30′ N) in Nanjing, Jiangsu Province in November 2022, and *T. miqueliana* seeds were collected from Huangzangyu National Forest Park in Anhui Province (117°06′ E, 34°06′ N) in November 2022. *P. subhirtella* and *J. mandshurica* seeds were collected from the campus of Nanjing Forestry University (118°48′ E, 32°06′ N) in June and October 2021, respectively. *P. sibirica* seeds were collected from Xiaolong Mountain, Tianshui City, Gansu Province (34°05' ~ 34°40′ N, 105°30′ ~ 106°30′E) in October 2022. All seeds were naturally dried indoors (temperature ranges from 3 to 17 °C, while the humidity was 58–70%). The empty seeds were removed by water separation and dried for later use. After harvesting, the experiment began within 1 month.

Experimental design

Since the six species are divided into endospermic and exalbuminous types, the endosperm treatment is required for the endospermic type (*T. miqueliana* and *T. henryana*), which involves many experimental factors, so the L_9 (3^4) orthogonal test method is adopted. The single-factor test method was used for the other four kinds of exalbuminous seeds. Each treatment evaluated 150 seeds per species (3×50), with staining conditions recorded at the conclusion of the test.

TZ staining test of T. miqueliana *and* T. henryana

The seeds were first soaked in deionized water for 48 h and then treated separately as follows: with a scalpel sterilized with alcohol, (1) the pericarp was removed (hulling), (2) after removing pericarp, the endosperm was cut in half along the cotyledon direction (longitudinal cut) or (3) the endosperm was cut in half vertically along the cotyledon direction (transverse cut) after removing pericarp, and then stained with TZ solution at 0.2, 0.5 or 1% concentration, respectively. The final distribution was placed in incubators at 30, 35 or 40 °C (in darkness) and stained for 80, 100 or 120 min, respectively. The orthogonal test was used to study the best TZ-staining method for *T. miqueliana* and *T. henryana* seeds (Cimbala, 2014). Four factors were set, including A, seed treatment method; B, TZ concentration (%); C, staining temperature (°C) and D, staining time (min), with three levels for each factor (Table 1).

Table 1. Orthogonal test scheme of TZ staining for *T. miqueliana* and *T. henryana* (L_9).

Factors			
Treatment method	TZ concentration (%)	Incubation temperature (°C)	Staining time (min)
Hulling	0.2	30	80
Longitudinal cut	0.5	35	100
Transverse cut	1	40	120

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TZ-staining test of J. mandshurica

The fresh *J. mandshurica* seeds were soaked in deionized water at room temperature (25 °C) for 36 h. Due to the complex seed structure, the seeds were cut along the middle suture (the midline of the two cotyledons). The tissues (radicles with a small part of the cotyledon) were taken out and soaked in 0.2, 0.5 or 1.0% TZ solution, then placed in incubators at different temperatures of 25, 30 or 35 °C and stained for 2, 4, 6 or 8 h in darkness.

TZ-staining test of S. tzumu

The fresh *S. tzumu* seeds were soaked in deionized water at 25 °C for 24 h. Then, the seed coat was removed and the embryos were taken out. The embryos were soaked in a TZ solution at concentrations of 0.2, 0.5 or 1.0%. Afterward, the embryos were placed in incubators at different temperatures of 30, 35or 40 °C. They were stained for 8, 12, 16 or 20 h in the darkness.

TZ test of P. subhirtella *and* P. sibirica

The fresh seeds of *P. subhirtella* and *P. sibirica* were soaked in water at 25 °C for 24 h, the seed coats were removed and embryos were extracted. Then the embryos were soaked in the TZ solution at concentrations of 0.2, 0.5 or 1.0% and placed in incubators at different temperatures of 25, 30 or 35 °C stained for 4, 6, 8 and 10 h in darkness.

Post-staining observation

After staining, the embryo and endosperm were observed anatomically and seeds were photographed for comparison (α7, Sony, Tokyo, Japan). Table 2 presents statistics and calculations of the performance of seed viability.

Verification of seed viability measurements

Additional 150 seeds (3 × 50) seeds of each species were used for germination trials as a point of comparison and verification for the TZ results. Seeds received the following dormancy-breaking treatments: (1) *T. miqueliana*, the seeds were soaked in H_2SO_4 for 15 min, then in GA_3 at a concentration of 0.5 g L^{-1} for 12 h, followed by stratification at 15°C for 60 d. (2) *T. henryana*, the seeds were soaked in H_2SO_4 for 15 min, then in GA_3 at a concentration of 1 g L^{-1} for 12 h, followed by cold stratification at 5°C for 45 d. (3) *J. mandshurica*, the seeds were first warm stratified at 20 °C for 30 d, and then cold stratified at 0°C for 60 d. (4) *P. subhirtella* and *P. sibirica*, the seeds were soaked in 1 g L^{-1} GA_3 for 12 h after the removal of the seed coat, followed by warm stratification at 20°C for 40 d. (5) *S. tzumu*, the seeds were soaked in 0.2 g L^{-1} GA_3 for 12 h, followed by cold stratification at 2°C for 120 d. Within 30 d, the germination was considered completed when no new seeds germinated for 7 consecutive days.

Table 2. Determination of the seed viability of *T. miqueliana and T. henryana* by TZ staining.

Viability level	Embryo staining	Endosperm staining
High	The embryo is bright red, more than 4/5 of the endosperm and the radicle is coloured	More than 1/3 of the endosperm is coloured pink or bright red
Medium	The embryo is bright red, more than 3/5 of the endosperm and the radicle is coloured	Less than 1/3 of the endosperm is coloured pink or bright red
Non-viable	The embryo is bright red or pink, less than 3/5 pigmented and the radicle is not pigmented	Endosperm is not stained or less stained

Phylogenetic tree construction of six species

To construct phylogenetic trees, internal transcribed spacer (ITS) sequence data from six species were compared and aligned using ClustalW software included in the MEGA package version 6.0.6 (El-Esawi et al., 2018). The aligned data set was analysed using the maximum likelihood (ML) to construct phylogenetic trees. According to the results of the Akaike Information Criterion (AIC) calculated in the MEGA package, the best model is selected for ML phylogenetic tree construction. The ML analysis is performed and the trees were constructed by calculating the initial tree (constructed by the BioNJ method) and selecting the Nearest-Neighbour Interchange (NNI) option for the following heuristic search. Bootstrap analysis was performed on 1000 replicates to calculate the support at the node. Bootstrap values are labelled on the nodes, and values less than 50 have been removed.

Determination of fat content

Fat content was determined by the Soxhlet extraction method (Botcha et al. 2011) using Solvent Extractor SER (VELP, Scientifica, Italy). First, 0.5 g of dry endosperm or cotyledon powder was wrapped with defatted filter paper and placed in a Soxhlet extractor. After the addition of petroleum ether, the extraction was carried out at a constant water temperature of 80°C for 16 h. The filter paper bag was then removed and dried in an oven at 105°C to volatilize the petroleum ether before being placed in a desiccator and cooled before weighing.

$$\text{Fat content } \% = \frac{W_1 + W_2 - W_3}{W_2} \times 100$$

where W_1 is the weight of filter paper (g), W_2 is the dry weight of endosperm (g) and W_3 is the weight of extracted endosperm (g).

Data analysis

Predictor variables (fat content, staining time, staining temperature and TZ concentration) and response variables (viability and germination rate) were tested. SPSS 25 software was used to calculate the mean and standard error. The Duncan test based on single-factor analysis of variance (ANOVA) and the between-subjects effect test were used to determine the significance of the results among the different treatments. All graphs were drawn using Origin Pro 2021 software (Origin Laboratory).

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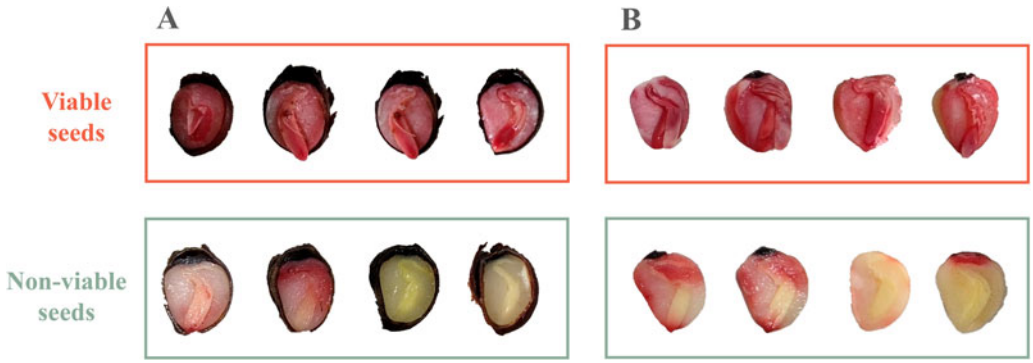

Figure 1. TZ staining diagram of seeds of *T. miqueliana* (A) and *T. henryana* (B).

Results

Determination of TZ-staining results of six hard-coated species

Standard for the interpretation of T. miqueliana *and* T. henryana *seed viability*

The two linden species have similar morphological characteristics, both are endospermic types and the outer layer is hard and poor permeability seed coat. Based on the TZ and germination results, we determined that seeds with staining of the embryo (≥80%), the radicle and the endosperm (≥33%) were highly viable; seeds with staining of the embryo (≥60%), the radicle and the endosperm (≤33%) were medium viable. Seeds where the embryo was not stained or sporadically stained and the endosperm was not stained were determined to be non-viable. (Fig. 1) (Table 2).

Standard for the interpretation of S. tzumu *seed viability*

S. tzumu seeds have no endosperm, so only the staining of the cotyledon and radicle needs to be observed. According to seed germination biology and the principle of the TZ test, the viable seeds were those in which the cotyledon and radicle stained bright red (Fig. 2A). The non-viable seeds were (1) the stained area of the cotyledon was less than 1/2 (Figs. 2B–2D); (2) the radicle and more than 1/2 of the cotyledon are stained, but the junction with the radicle is defective (Figs. 2E and 2F); (3) the cotyledon is completely stained but the radicle is not (Fig. 2G); (4) the radicle is not stained and the area of the cotyledon is less than 1/2 (Fig. 2H) and (5) neither the cotyledon nor the radicle was stained (Fig. 2I).

Standard for the interpretation of J. mandshurica, P. subhirtella *and* P. sibirica *seed viability*

The seeds of *J. mandshurica*, *P. subhirtella* and *P. sibirica* are all albuminous seeds, so their viability is determined by embryo staining. At the end of the staining, the viability of the seed is judged according to the part of the seed that is stained, the size

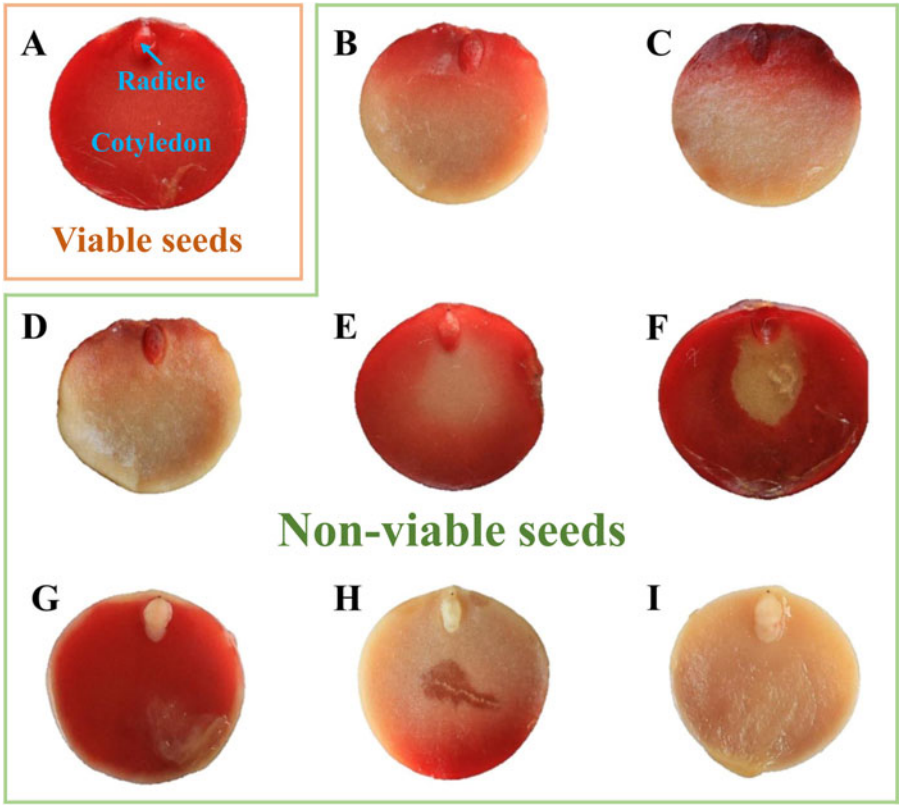

Figure 2. Staining schematic diagram of *S. tzumu* seed viability. (A) Viable seeds and (B–I) non-viable seeds. Note that (A) the cotyledon and the radicle were stained bright red; (B–D) the stained area of the cotyledon was less than 1/2; (E and F) the radicle and more than 1/2 of the cotyledon are stained, but the junction with the radicle is defective; (G) the cotyledon is completely stained but the radicle is not; (H) the radicle is not stained and the area of the cotyledon is less than 1/2; (I) neither the cotyledon nor the radicle was stained.

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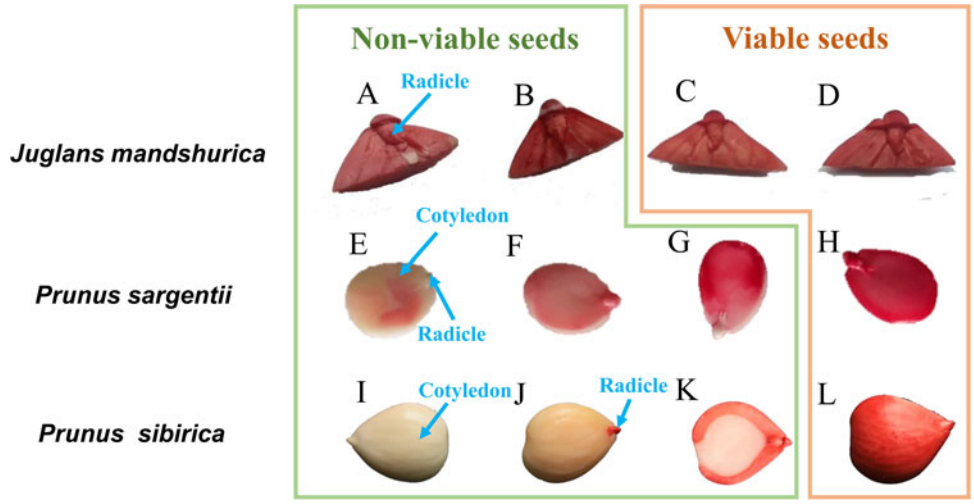

Figure 3. Staining schematic diagram of *J. mandshurica* (A–D), *P. subhirtella* (E–H) and *P. sibirica* (I–L) seed viability. Note that (D, H and L) radicle and cotyledon all are stained; (C) radicle stained, most of the cotyledon stained; (J) radicle stained but cotyledon not stained; (B and G) radicle not stained and cotyledon partially stained; (E and I) neither radicle nor cotyledon stained. (A and K) The cotyledon and radicle are stained, but the hypocotyl is not stained. (F) Both the radicle and the cotyledon are stained, but the staining is light.

of the stained area and the degree of staining. Specific scoring criteria are as follows:

(1) *Viable seeds*: radicle and cotyledon all stained (Fig. 3D, 3H, and 3L); radicle stained, most of the cotyledon stained (Fig. 3C);
(2) *Non-viable seeds*: radicle stained but cotyledon not stained (Fig. 3J); radicle not stained and cotyledon partially stained (Figs. 3B, and 3G,); neither radicle nor cotyledon stained (Fig. 3E, and 3I). The cotyledon and radicle are stained, but the hypocotyl is not stained (Fig. 3A, and 3K). Both the radicle and the cotyledon are stained, but the staining is light (Fig. 3F).

TZ-staining test results of six hard-coated species

TZ-staining results of the endospermic type

The orthogonal test was used to investigate the optimal TZ-staining method for *T. miqueliana* and *T. henryana* seeds. The results are presented in Tables 3 and 4. Different pre-treatment and TZ staining methods had significant differences in the seed viability determination results. The lowest viability level of *T. henryana* seeds

Table 3. TZ-staining results of *T. henryana* seed.

Treatment	[A] Treatment method	[B] TZ concentration (%)	[C] Incubation temperature (°C)	[D] Staining time (min)	Seed viability (%)
1	Hulling	0.2	30	80	60.00c
2	Hulling	0.5	35	100	75.00b
3	Hulling	1	40	120	96.67a
4	Longitudinal cut	0.2	30	100	38.33e
5	Longitudinal cut	0.5	35	80	51.67cd
6	Longitudinal cut	1	40	120	75.00b
7	Transverse cut	0.2	30	100	46.67de
8	Transverse cut	0.5	35	120	63.33c
9	Transverse cut	1	40	80	55.00cd
*k*1	0.772	0.483	0.483	0.556	
*k*2	0.550	0.633	0.633	0.656	
*k*3	0.550	0.756	0.756	0.661	
R	0.222	0.272	0.272	0.106	
df	2	2	2	2	
F	1.220	1.364	1.364	2.905	
P	0.353	0.283	0.283	0.029*	

Note: The different lowercase letters after the values in the same column indicate significant differences between treatments ($P \leq 0.05$). The '*' indicates a significant difference between treatments ($^*P \leq 0.05$). *k*1, *k*2 and *k*3 represent the average seed viability of each factor at each level. *R* represents 'range', which indicates the magnitude of the effect of each factor on the result.

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Table 4. TZ-staining results of *T. miqueliana* seed.

Treatment	[A] Treatment method	[B] TZ concentration (%)	[C] Incubation temperature (°C)	[D] Staining time (min)	Seed viability (%)
1	Hulling	0.2	30	80	0.00e
2	Hulling	0.5	35	100	66.00c
3	Hulling	1	40	120	61.33c
4	Longitudinal cut	0.2	30	100	86.00b
5	Longitudinal cut	0.5	35	80	82.00b
6	Longitudinal cut	1	40	120	98.33a
7	Transverse cut	0.2	30	100	14.00d
8	Transverse cut	0.5	35	120	10.00d
9	Transverse cut	1	40	80	17.33d
*k*1	0.424	0.378	0.378	0.364	
*k*2	0.942	0.560	0.560	0.569	
*k*3	0.138	0.567	0.567	0.571	
R	0.518	0.189	0.189	0.207	
df	2	2	2	2	
F	6.723	3.435	3.435	7.187	
P	0.007**	0.022*	0.022*	0.001**	

Note: The different lowercase letters after the values in the same column indicate significant differences between treatments ($P \leq 0.05$). The '*' indicates a significant difference between treatments (*, $P \leq 0.05$ **, $P \leq 0.01$). k1, k2 and k3 represent the average seed viability of each factor at each level. R represents "range", which indicates the magnitude of the effect of each factor on the result.

determined by TZ staining was 38.33% and the highest was 96.67%, and the staining effect of treatment 3 (hulling, 1%, 40 °C, 120 min) yielded the highest measure of viability. The lowest TZ staining rate of *T. miqueliana* seeds was 0, while the highest was 98.33%, and the staining effect of treatment 6 (longitudinal cut, 1%, 40 °C, 120 min) yielded the highest measure of viability. ANOVA and range analysis (*R*-value) showed that the factors affecting the determination of TZ staining of *T. henryana* seeds were as follows: TZ concentration = staining temperature > treatment method > staining time. However, the viability of *T. henryana* seed was not significant for the first three factors ($P > 0.05$), but only for staining time ($P < 0.05$). The factors affecting the determination of TZ staining of *T. miqueliana* seeds were as follows: treatment method > staining time > TZ concentration = staining temperature. All the four factors had significant effects on the TZ-staining level of *T. miqueliana* seeds. According to the results of the ANOVA and range analysis, the best TZ-staining method for *T. henryana* was treatment 3 (hulling, 1%, 40 °C, 120 min), and the best method for staining the seeds of *T. miqueliana* was treatment 6 (longitudinal cut, 1%, 40 °C, 120 min).

TZ-staining results of the exalbuminous type

The results of different staining temperatures, TZ concentrations and staining times on the viability assessment of four kinds of exalbuminous hardiness seeds are shown in Tables 5 and 6. Staining temperatures, TZ concentrations and staining times all had significant effects on the results ($P < 0.01$). The number and the degree of stained seed increased with increasing staining time, and the overall performance being that the higher the TZ concentration, the shorter the staining time (Table 5). The four exalbuminous hard seeds required at least more than 6 h of staining time, and all required a 1% TZ concentration and a staining temperature above 35 °C to achieve the best staining effects and viability determination (Table 6).

Excessive long TZ staining time will lead to an excessive deep staining degree, resulting in misjudgement of seed viability level. In the *P. subhirtella* staining experiment, the most accurate staining information could be obtained when the seeds were soaked in 1% TZ for 8 h. After 10 h, the staining degree was too deep, which was not conducive to the interpretation of viability level. There is a similar phenomenon in the staining process of *P. sibirica* seeds (Table 5). Too high concentration of TZ concentration and too long staining time will lead to darker red colour and staining of all parts, and this is not conducive to obtain accurate information on seed viability. For the determination of seed viability and TZ-staining test results, the optimal TZ-staining conditions for *S. tzumu* seeds are as follows: at 35 °C, the seeds were immersed in TZ solution with 1% concentration, then stained for 20 h and the final viability was determined to be 84%. The optimal TZ-staining conditions of *J. mandshurica* seeds were as follows: the seeds were immersed in the TZ solution with 1% concentration at 35 °C and then stained for 6 h, and the final viability was determined to be 96%. The optimal TZ-staining conditions for *P. subhirtella* seeds were as follows: the seeds were immersed in the TZ solution with 1% concentration at 35 °C, then stained for 8 h and the final viability was determined to be 94%. The optimal TZ-staining conditions for *P. sibirica* seeds were as follows: the seeds were immersed in the TZ solution with 1% concentration at 35 °C, then stained for 8 h and the final viability was determined to be 96% (Table 6).

Reliability verification of TZ staining

Six kinds of species in different replicates were selected for germination after dormancy release. As shown in Fig. 4, the seed

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Table 5. Effects of different TZ test conditions on the viability of four kinds of exalbuminous hard seeds.

TZ-staining results of *S. tzumu* seed											
Incubation temperature (°C)	TZ concentration (%)	Staining time (h)	Seed viability (%)	Incubation temperature (°C)	TZ concentration (%)	Staining time (h)	Seed viability (%)	Incubation temperature (°C)	TZ concentration (%)	Staining time (h)	Seed viability (%)
30	0.2	8	0.00m	35	0.2	8	0.00m	40	0.2	8	14.00g
		12	0.67m			12	4.00m			12	46.67 e
		16	1.33m			16	20.67 j			16	57.33 d
		20	2.67m			20	22.00 ij			20	62.67 cd
	0.5	8	0.00m		0.5	8	2.00m		0.5	8	35.33 f
		12	2.67m			12	22.00 ij			12	66.67 cd
		16	8.00l			16	50.00 e			16	72.00 c
		20	18.67 j			20	62.00 c			20	80.67 b
	1	8	0.67m		1	8	20.00 j		1	8	45.33 e
		12	3.33m			12	25.33 hi			12	71.33 c
		16	12.67 k			16	70.00 b			16	80.33 b
		20	26.67h			20	74.00 a			20	88.67 a
TZ staining results of *J. mandshurica* seed											
Incubation temperature (°C)	TZ concentration (%)	Staining time (h)	Seed viability (%)	Incubation temperature (°C)	TZ concentration (%)	Staining time (h)	Seed viability (%)	Incubation temperature (°C)	TZ concentration (%)	Staining time (h)	Seed viability (%)
25	0.2	2	12.00 i	30	0.2	2	8.00 j	35	0.2	2	32.00 f
		4	18.00h			4	17.67 hi			4	34.33 f
		6	19.67h			6	20.00h			6	42.00 e
		8	24.00 gh			8	26.00g			8	44.33 e
	0.5	2	22.00 gh		0.5	2	38.00 e		0.5	2	28.00g
		4	32.00 f			4	42.00 e			4	34.33 f
		6	36.33 ef			6	56.00 d			6	42.33 e
		8	44.00 e			8	60.00 c			8	68.00 bc
	1	2	48.67 de		1	2	38.67 e		1	2	48.00 de
		4	52.00 d			4	64.00 c			4	76.00 b
		6	51.33 d			6	71.33 b			6	98.00 a
		8	54.00 d			8	74.00 b			8	98.67 a
25	0.2	4	8.00m	30	0.2	4	23.67 jk	35	0.2	4	41.33g

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		6	16.00 k			6	24.00 jk			6	48.00 f
		8	19.67 jk			8	32.00 i			8	63.33 d
		10	27.67 j			10	43.67g			10	74.00 c
	0.5	4	17.33		0.5	4	26.33 j		0.5	4	42.33g
		6	22.00 k			6	36.00h			6	51.67 ef
		8	31.33 i			8	53.67 e			8	60.00 d
		10	37.33h			10	62.33 d			10	72.00 c
	1	4	34.33h		1	4	54.00 e		1	4	74.00 c
		6	54.00 e			6	76.33 c			6	82.00 b
		8	71.33 c			8	82.00 b			8	94.00 a
		10	76.00 c			10	86.00 b			10	98.33 a
TZ staining results of *P. sibirica* seed											
Incubation temperature (°C)	TZ concentration (%)	Staining time (h)	Seed viability (%)	Incubation temperature (°C)	TZ concentration (%)	Staining time (h)	Seed viability (%)	Incubation temperature (°C)	TZ concentration (%)	Staining time (h)	Seed viability (%)
25	0.2	4	14.33 i	30	0.2	4	20.67 f	35	0.2	4	45.33 e
		6	37.33g			6	32.00 e			6	56.67 d
		8	34.00g			8	78.00 c			8	77.33 c
		10	42.00 f			10	81.33 bc			10	82.67 bc
	0.5	4	31.33 gh		0.5	4	66.00 d		0.5	4	25.33 f
		6	42.00 f			6	72.33 c			6	76.67 c
		8	78.00 c			8	78.33 c			8	86.00 b
		10	86.67 b			10	88.33 ab			10	88.67 b
	1	4	32.00g		1	4	61.67 d		1	4	52.33 d
		6	65.67 d			6	84.33 b			6	87.33 b
		8	77.33 c			8	88.00 ab			8	95.33 a
		10	88.33 ab			10	90.77 a			10	97.67 a

Note: The different lowercase letters after the values in the same column indicate significant differences between treatments ($P \leq 0.05$).

Table 6. Optimal TZ-staining method for hard seeds of six species.

Types of seed	Species	TZ-staining conditions			Seed viability (%)
		Incubation temperature (°C)	TZ concentration (%)	Staining time (h)	
Endospermic seeds	*T. henryana*	40	1	2	96.67
	T. miqueliana	40	1	2	98.33
Exalbuminous seeds	*S. tzumu*	40	1	20	84
	J. mandshurica	35	1	6	96
	P. subhirtella	35	1	8	94
	P. sibirica	35	1	8	95.33

viability level of six kinds of seed species determined by the optimal TZ staining method was slightly higher than the actual germination rate, but the difference was not significant. In all cases, significant differences in viability were in agreement with significant differences in germination, confirming the reliability of the best TZ method for seed viability of six species.

Phylogenetic relationship of six species

It can be seen from the phylogenetic diagram (Fig. 5) that although the six species are all hard seeds, *S. tzumu* is the most distantly related. *T. miqueliana and T. henryana* are closely related, and both have endosperm seeds, so the staining time is shorter than that of the other four plants. The ITS sequence of

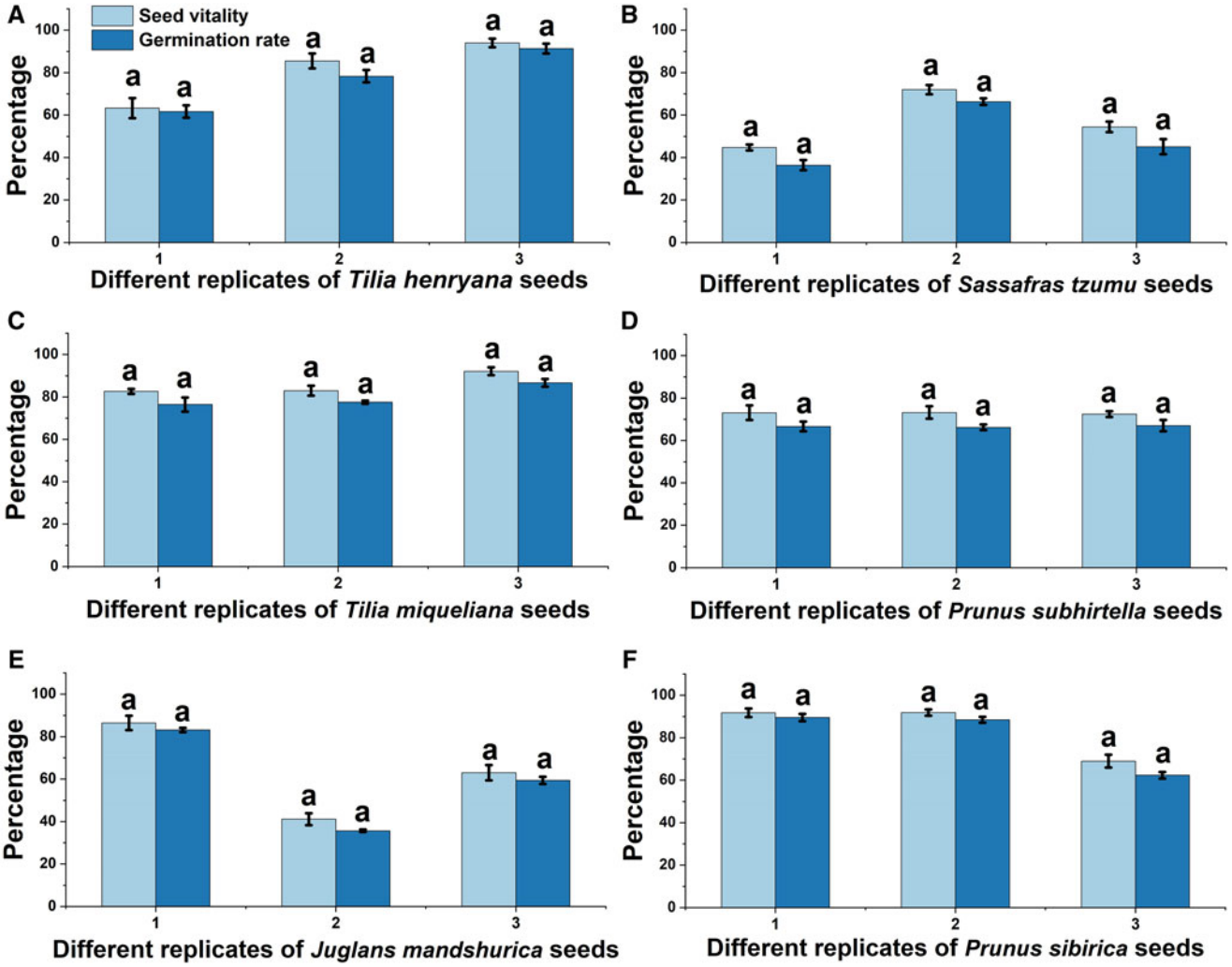

Figure 4. Comparison of viability measured by TZ staining with germination of (A) *T. henryana*; (B) *S. tzumu*; (C) *T. miqueliana*; (D) *P. subhirtella*; (E) *J. mandshurica* and (F), *P. sibirica*. Notes: Lowercase letters in the same replicate indicate no significant difference at the 0.05 level.

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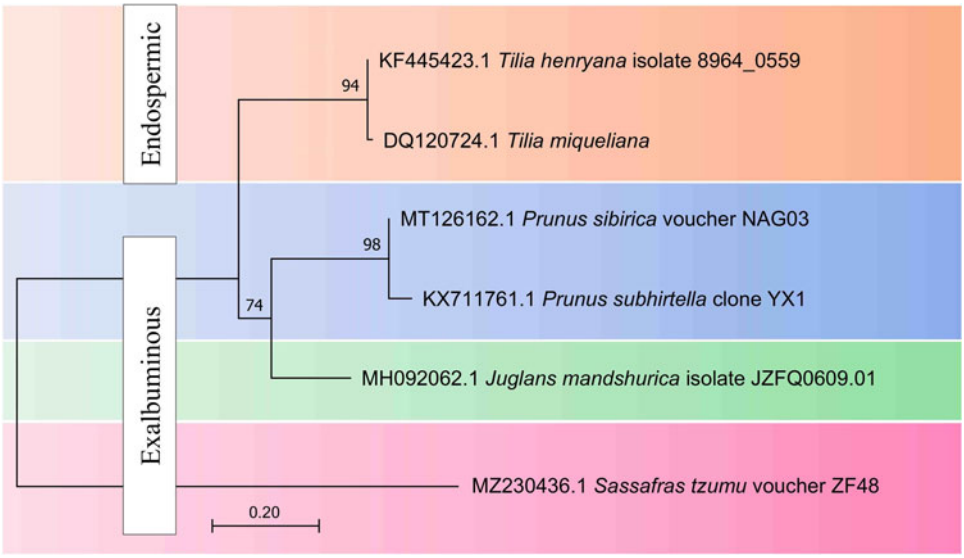

Figure 5. ML phylogeny of ITS regions for the study species. ML phylogeny of ITS regions for the study species. Notes: *S. tzumu* voucher ZF48 was chosen as out-group. Support in nodes is indicated above branches and is represented by bootstrap values. Bootstrap values lower than 50 is hidden. The best-fit model of ML phylogeny according to AIC: Tamura 3-parameter (T92) + G; alignment ITS = 551 bp. Scale bar: 0.20 substitutions per nucleotide position.

T. henryana isolate 8964_0559 clustered with *T. miqueliana* (bootstrap = 94%) (Fig. 5). The other three dicotyledonous plants are relatively closely related, ITS sequence of *J. mandshurica* isolate JZFQ0609.01 clustered with *P. sibirica* voucher NAG03 and *P. subhirtella* clone YX1 (bootstrap = 74%) (Fig. 5). They also have a high degree of similarity in the staining conditions, except for the difference of 2 h in the staining time; other conditions are completely identical (Table 6).

Notes: S. tzumu voucher ZF48 was chosen as out-group. Support in nodes is indicated above branches and is represented by bootstrap values. Bootstrap values lower than 50 are hidden. The best-fit model of ML phylogeny according to AIC: Tamura 3-parameter (T92) + G; alignment ITS = 551 bp. Scale bar: 0.20 substitutions per nucleotide position.

Seed fat content of six species

All six species had high fat content, but the range of fat content was quite variable, ranging from 28.4 to 67.8% (Fig. 6A). The seeds of *T. miqueliana* and *T. henryana* had the lowest fat content, which was 28.4 and 29.6%, respectively. The seeds of *P. subhirtella* and *P. sibirica* were the next, with 47.9 and 48%, respectively, and the seeds of *S. tzumu* had the highest fat content of 67.8%. There was a correlation between seed fat content and staining conditions such as incubation temperature, TZ concentration and staining time (Fig. 6B). The higher the fat content in seeds, the lower the incubation temperature and TZ concentration required for staining, and the longer the staining time.

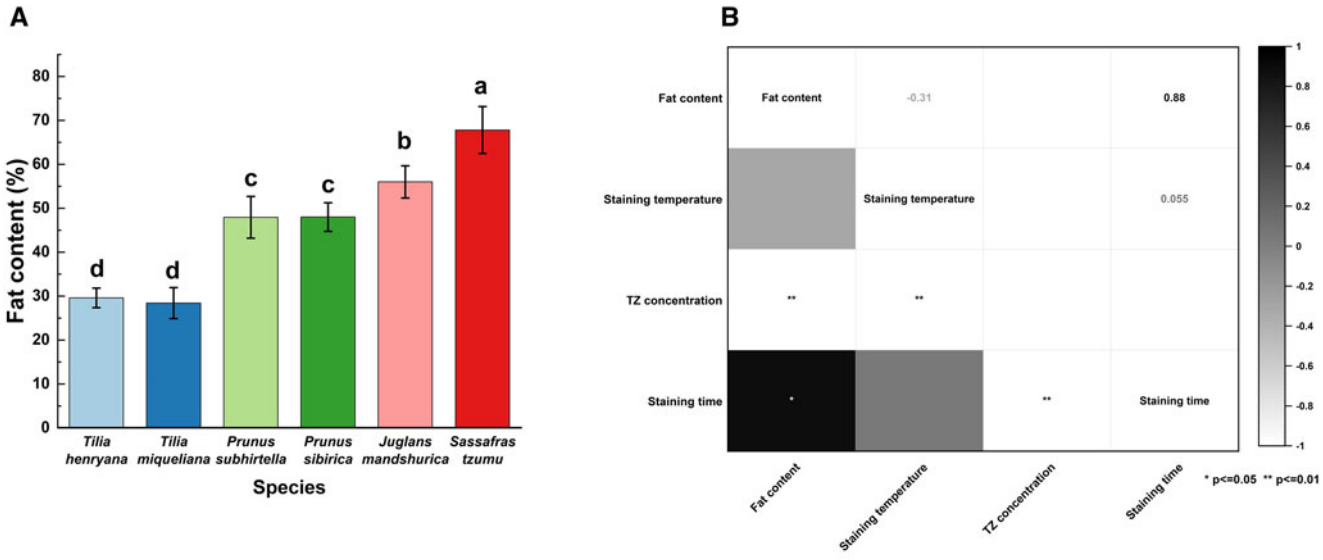

Figure 6. The content of fat in the endosperm or cotyledons of six species (*T. miqueliana*, *T. henryana*, *S. tzumu*, *P. subhirtella*, *P. sibirica* and *J. mandshurica*) (A). The correlation between fat content, incubation temperature, TZ concentration, staining time and seed viability (B). *Note*: The different lowercase letters indicate significant differences between treatments ($P \leq 0.05$). The '*' indicates a significant difference between treatments (*$P \leq 0.05$; **$P \leq 0.01$).

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Discussion

Analysis of TZ-staining results

Seed viability is an important index to evaluate the suitability of seed storage methods and sowing quality (Gough, 2020). Different seeds require different TZ staining methods to obtain the best-staining effect (Zhang et al., 2015). The seed coats of the six kinds of hard seeds are hard and have poor permeability, all of them have the characteristics of deep dormancy, which makes the viability assessment of these seeds difficult. At the same time, these seeds have relatively high requirements for TZ solution concentration and need to be stained in 1% TZ solution, which may be related to the dormancy characteristics of the seeds. The results of this study showed that the optimized TZ detection method could accurately determine the viability of six species of hard-coated seeds. The staining conditions of *S. tzumu* are also very different from those of the other five plants, and the staining time is 20 h. In addition to *S. tzumu*, the remaining five species of hard seeds do not stain for more than 8 h.

The reason why the germination percentage is lower than the viability of seeds may be that the seeds need to be treated with acid corrosion and stratification during the dormancy release process and some seeds with low vitality may rot and deteriorate during stratification (Kozlowski and Pallardy, 1997; Hirano et al., 2005; Meyer, 2006). Airborne bacteria or fungi can also cause infection during the dormancy and germination process (Bradbeer, 2013). In addition, seed dormancy and the presence of germination inhibitors may also affect the final germination percentage. It is particularly noteworthy that it is useful to determine the viability of seed species that are hard to germinate and have deep dormancy. Examples are tree, shrub and grass seeds, as well as certain crops during the first few months after harvest in some crops when the dormancy is at its highest level (e.g., *Kentucky bluegrass*) (Elias et al., 2006). As the same time, when speed is important and quick decisions about the viability levels of a seed lot has to be made on a short notice, whether the seeds are dormant or non-dormant. Compared with the long-term and laborious dormancy release treatment required for the germination of hard seeds (Jones et al., 2016), the optimal TZ-staining method obtained in this study can accurately and quickly evaluate the viability of different batches of seeds.

TZ-staining characteristics of hard seeds

Incubation temperature is one of the important factors determining the effectiveness of TZ staining. In this study, all the hard seeds of the six species required a staining temperature above 35 °C during the TZ-staining process, indicating that the staining temperature was higher than that of conventional seeds (Li et al., 2022). This is similar to rice and *Vernicia fordii* seeds. The incubation temperature is the most critical factor for the TZ-staining effect of rice seeds and *V. fordii* seeds. Chen et al. (2021) found that at an incubation temperature of 20°C, the staining percentage of rice seeds was only 43%. As the incubation temperature increased, the staining percentage increased gradually. The staining percentage increased to 75% at 30°C and further to 81% at 40° C. Gu et al. (2020) found that the staining percentage of *V. fordii* embryos increased with increasing incubation temperature. The staining percentage was 0% at both 20 and 25°C. When the temperature reached 40°C, the staining percentage was 68.89% and continued to increase to 71.11% at 45°C. It shows that the seeds all have dormancy characteristics and belong to the north temperate zone seeds. The internal permeability enhancement and physiological activation of the seeds require higher temperature.

TZ staining requires the TZ solution to access the seed tissues in order to activate respiratory enzymes to release hydrogen ions. This allows the TZ solution to access the internal tissues of the seed. The hydrogen ions reduce the colourless TZ solution to red formazan, which stains living tissues with red colour, while dead tissues remain unstained (Elias et al., 2006). The hydration rate of the TZ solution entering the seed and reacting with the tissues is therefore critical. Lipids may be broadly defined as hydrophobic (Fahy et al., 2009). Interestingly, we found that the fat content of these six species is related to the TZ-staining time. In addition, fat content was similar within the same genus, and the more distant the genetic relationship, the greater the difference in fat content and the higher the fat content the longer the staining time required. Munz et al. (2017) found that during the imbibition process of *B. napus* seeds, the storage lipids accumulated in the endosperm play the role of an efficient hydrophobic barrier between the embryo and the integuments. It can be seen that the fat content in seeds is one of the important factors affecting the TZ-staining speed.

Association between plant species relatedness and TZ-staining conditions in seeds

The species of the same genus or even the same family may have great differences in staining conditions, so it is necessary to adjust the method of each specific species when performing TZ-staining test on seeds (Lamarca and Barbedo, 2014). The closely related species have similar staining characteristics due to the similarity in morphology (Adams et al., 2005; Martín-Gómez et al., 2020), composition and so on. *T. miqueliana and T. henryana* belong to the genus Tilia, except for different seed treatment methods, the staining conditions are completely the same, so that other species of the same genus may also have similar TZ-staining conditions. In addition, *P. subhirtella* and *P. sibirica* have a closer relationship and are completely consistent in various TZ-staining conditions. Although *J. mandshurica* do not belong to the same genus as *P. subhirtella* and *P. sibirica*, it still belongs to a similar position in the phylogenetic tree and has similar staining conditions. However, there are differences in the staining time. The staining conditions of *S. tzumu* are very different from those of the above species, and the staining time takes 20 h, which is far more than that of the other five kinds of hard seeds. In addition, there are also great differences in the impact of staining temperature and TZ concentration. According to the location of different species in the evolutionary tree, TZ-staining conditions of species with similar relatives can be inferred, which can save a lot of time and cost and human and material resources. At the same time, the more species that know TZ-staining conditions, the more accurate and detailed the prediction will be. The establishment of a staining condition model based on the available information and the rapid prediction of the staining conditions of unknown species are of great help for the conservation and utilization of germplasm resources, especially for the rapid detection of vigour of hard seeds.

Conclusion

The TZ staining of six species of hard seeds requires a staining temperature above 35 °C, and the TZ solution concentration is

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above 1%. Therefore, it provides evidence that that the hard seeds in the northern temperate zone generally require a higher staining temperature and TZ concentration. In addition to these shared characteristics, it is important to note that the six hard seeds require different optimal stain treatments. Endospermic seeds, for example, require a shorter staining time compared to exalbuminous seeds. The higher the fat content in seeds, the lower the incubation temperature and TZ concentration are required for staining, and the duration of the staining process increases as the temperature and TZ concentration increase. The closer the relationship between the two species, the more similar their staining conditions are. The TZ-staining method of similar species can be predicted according to phylogenetic proximity, and the viability of new species can be detected quickly.

Data availability. All data are presented in the article.

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Author contributions. Y.B.S. conceived the original screening and research plans; C.Y.P. and Y.W. designed and performed all experiments; W.H.H., Z.Y.D., X.R.S., M.Z.W., J.Y.X., X.Y.Y. and C.C.G. analysed the data; C.Y.P. and Y.W. wrote the article with contributions of all the authors. Y.B.S. and H.W.P. revised the article. Y.W. agrees to serve as the author responsible for contact and ensures communication. All authors have read and approved the manuscript.

Competing interest. The authors declare that they have no significant competing financial interests or personal relationships that could have appeared to influence the work described in this manuscript.

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