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Physiological dormancy and dormancy release of *Sassafras tzumu*, a colored-leaf tree species with high landscape and economic value

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***Sassafras tzumu* is one of the most valuable tree species in southern China. However, the dormancy of the seed limits its seedling quality. In order to improve the germination percentage of seeds, characteristics, causes of dormancy, changes in endogenous hormones and nutrients during storage were investigated. The results showed that seed viability was 78.11%, and the highest water absorption rate was 26.09%. The germination percentage of the embryo without cotyledon was 21.13%, but intact seed and embryo could not germinate on hormone-free medium. Seed embryo and coat extracts were found to have a significant inhibitory effect on seed germination. Germination percentage were significantly positively correlated to free amino content ($p < 0.01$) and negatively correlated to soluble sugar content ($p < 0.05$) during storage. The levels of GA_3 and GA_3/ABA had significant effects on the germination percentage of *S. tzumu* seed storage at 4°C. The results suggest that *S. tzumu* seeds are in intermediate physiological dormancy at maturity and are mainly caused by the presence of inhibitory substances in the seed tissues. Furthermore, changes in endogenous hormones and metabolism of nutrients at 4°C can significantly promote the release from dormancy of *S. tzumu* seeds.**

Keywords: *Sassafras tzumu*, Seed Dormancy, Wet Sand Storage, Phytohormones, Nutrients

Introduction

Seeds are the main units of dispersal of most plants and allow colonization of new geographic areas, which is of great significance to the continuation of species (Arc et al. 2013, Feng et al. 2018). To regenerate successfully, it is essential for plants to produce viable seeds (Wotton 2018). The germination of seed is crucial to the propagation of plant species (Rajjou et al. 2012, Liang et al. 2020). To reduce the impact of adverse environments, seeds of many species are dormant until environmental conditions become favourable, which is a trait exhibited by newly produced seeds (Finch-Savage & Leubner-Metzger 2006, Kranner et al. 2010, Lee & Lopez-Molina 2013).

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Based on numerous studies, it is obvious that many seeds are dormant at maturity. A freshly matured dormant seed (or other germination unit) is said to have primary dormancy, which develops during seed maturation on the mother plant (Baskin & Baskin 2004). In addition to the environment factors, the endogenous factors such as embryo development, seed coat and phytohormones also play an important role in seed dormancy. The seed coat has been shown to regulate permeability to water, oxygen, or germination inhibitor, and is closely related to seed permeability in many species (Serrano et al. 2014, Acosta et al. 2020, Zemouri et al. 2020). Generally, when the seed coat is removed, respiration and water absorption are enhanced. Seeds with testa-imposed dormancy will germinate following damage or removal of the testa (Finkelstein et al. 2008). Phytohormones play a key role in seed development processes such as maturation, dormancy and germination. The balance between abscisic acid (ABA) and gibberellic acid (GA) is considered to be the key to seed dormancy and germination, but not the absolute concentration or content of ABA and GA (Arc et al. 2013, Deng et al. 2016, Shu et al. 2016). Generally, bioactive GA contributes to embryo development (Lulsdorf & Ying 2013), and ABA is the most prominent germination-inhibiting hormone in dormancy and germination control (Chen et al. 2008, Bian et al. 2018). The sharp changes of indole acetic acid (IAA) are recognized as one of the first signals leading to embryogenesis (Thomas et al. 2002) and inhibition of seed germination of seeds to a certain degree (Ramaih et al. 2003, Liu et al. 2013). The Zeatin riboside (ZR) has also been confirmed to be closely related to seed dormancy and germination (Yan et al. 2017). In addition, each phytohormone has a specific effect on the germination process, either acting alone or working together.

Sassafras tzumu is one of the most important species in the south of China (Chen et al. 2020), and it has begun to come into focus as a colored-leaf tree in recent years (Li et al. 2019). The *S. tzumu* is propagated from seeds, however, it is considered that the seeds of *S. tzumu* are dormant at maturity (Zhou et al. 2006). Therefore, a suitable preservation technology is essential to maintain seed vitality and ensure the continuous supply of viable seeds. Dormancy release by cold stratification has been reported in many species, especially those with physiologically dormant seeds (Baskin & Baskin 2004, Wotton 2018, Yang et al. 2019). In production practice, cold stratification for 8 months can release the dormancy of *S. tzumu* seeds and more than 90% of embryo release the dormancy under 0-5 °C (Xiao & Zhou 1988, Zhou et al. 2006). However, the effects of cold stratification on dormancy release and physiological characteristics of *S. tzumu* are still unclear. To improve the understanding of dormancy in *S. tzumu* seeds, three questions would be discussed in this study: (i) what are the characteristics of *S. tzumu* seeds? (ii) What are the causes of seed dormancy and the

type of dormancy? (iii) What preparation for seed dormancy release and germination during storage? The research will provide some new insights and support for the reproduction of *S. tzumu*.

Materials and methods

Study area and species

S. tzumu seeds were collected from the Research Institute of Subtropical Forestry, Chinese Academy of Forestry, Zhejiang, Hangzhou, China (119° 57′ E, 30° 03′ N). The mature fruits were collected in batches from June to July in 2018. Seeds were collected after removing the pericarp and impurities.

Seed characteristics

Seeds were randomly selected by quartering and the color of the seed coat was assessed. The vertical and horizontal diameter of the seeds were measured using a Vernier caliper. One hundred seeds were weighed to calculate the 1000-grain weight. For initial seed moisture measurement, about 5 g seeds were dried in an oven at 103 °C until constant weight, and the percentage of moisture was calculated based on fresh weight (Baladi & Balouchi 2016). The seeds were cut longitudinally and placed in 0.4% 2,3,5-triphenyl-tetrazolium chloride (TTC) solution for 3 h at 30 °C, and the seeds with pink or red embryos indicated viability (Liang et al. 2020). Intact seeds were placed in a 100-mL beaker, soaked with distilled water, and allowed to swell at 25 °C. The seeds were weighted for six times every 2 h and then weighted every 4 h until constant weight. All measurements were repeated thrice. The water absorption rate (WAR, %) and water uptake rate (WUR, g h^{-1}) were determined by using the following equations (eqn. 1, eqn. 2):

$$WAR=\frac{(W_n-W_i)}{W_i}\cdot 100 \quad (1)$$

$$WUR=\frac{W_n-W_{n-1}}{T_n-T_{n-1}} \quad (2)$$

where W_i represent the initial weight of seeds, W_n is the weight of seed at weighed *n*-th, and T_n is the processing time at weighed *n*-th.

Germination test

The mature seeds were placed in sand: leaf mold (1:3) at 15 °C, 20 °C, 25 °C, 30 °C, 35 °C, 25 °C/15 °C, 30 °C/15 °C, 30 °C/20 °C (14h/10h, light/dark), and the germination percentage was recorded 60 days (d) later, respectively.

To determine which tissues contributed to prevent germination in dormant *S. tzumu* seeds, different seed tissues were sequentially removed and the germination percentage was evaluated. Seeds were sterilized with 70% ethanol for 1 minute, 2% sodium hypochlorite for 10 minutes, followed by washing four times in sterile water. The intact seed, embryo (seed without coat) and embryo without cotyledon were inoculated on Murashige-Skoog (MS) medium. Both the hormone-free MS medium and the MS with 1 g L^{-1} GA_3 were used, which were supplemented with 3% sucrose and 0.8% agar at pH 5.8. All cultures were incubated in a chamber at 25 °C ± 1 °C with 10 h dark and 14 h light, and the germination percentage was recorded 60 days later. Each experiment was repeated with 100 seeds thrice.

Bioassay of seed extracts

The seed coat and embryo parts with a weight of 200 g were separately grounded, and then extracted with 500 mL of 80% methanol at 4 °C for 24 h. This extraction process was repeated once. The extract solution was successively extracted by petroleum ether, ether, ethyl acetate and methanol. All extract solutions were concentrated to 100 mL. The *Brassica campestris* seeds were used to test the germination inhibitory substances activity (Bian et al. 2018, Gao et al. 2020). Two pieces of filter paper were placed in each petri dish, and 3 mL of the extracts were added to the dishes. When the organic solvent was completely volatilized, approximately 3 mL of water was added to each petri dish. The *B. campestris* seeds were placed in petri dishes and germinated in a phytotron at 25 °C. The germination percentage was calculated after 48 h. Seedling height and root length were measured after 72 h. Each experiment with 100 seeds was repeated thrice.

Treatment and preservation of seeds

Seeds were mixed with wet sand (disinfected with 0.5% $KMnO_4$) in the proportion of 1:3, and packed in self-sealed bags (25 × 17 cm). Three holes were cut to maintain enough air permeability and stored at 4 °C in refrigerator for 240 days, whereas the control was stored at 25 °C (three replicates). The sands were turned over and the right amount of distilled water was added every 15 days to maintain moisture. The seeds were taken out after storage for 0 d (S0), 60 d (S60), 120 d (S120), 180 d (S180), and 240 d (S240) and stored at -80 °C after frozen in liquid nitrogen until analysis.

Endogenous hormones and seed reserves

Samples (1.0 g) using 10 mL of 80% (v/v) pre-cooling methanol at 4 °C for 12 h in dark, followed by centrifuging at 10,000×g at 4 °C for 15 min (Bian et al. 2018). The final 2 μL of filtrate solution was used for the quantification of hormones using an ultra-performance liquid chromatography system (model 1290 Infinity®, Agilent Corp., Santa Clara, CA, USA). The content of ABA, GA_3, IAA and ZR was determined using the external standard method and was expressed as μg g^{-1} FW (Yang et al. 2019).

Soluble sugar content was measured by using anthrone colourimetry (Ni et al. 2018). The seeds were transferred to a pre-cooled mortar and ground into a power before being mixed with 10 mL phosphate buffer solution (0.05 mol L^{-1}, pH=7.8) that had been kept on ice. The homogenate was centrifuged and the soluble protein was measured by Coomassie brilliant blue G-250 staining method (Xue et al. 2015). After seeds were ground, concentration of fat was determined by the Soxhlet extraction method (Soriano et al. 2011). The free amino acid of seeds was measured by using ninhydrin. Three biological replications were performed.

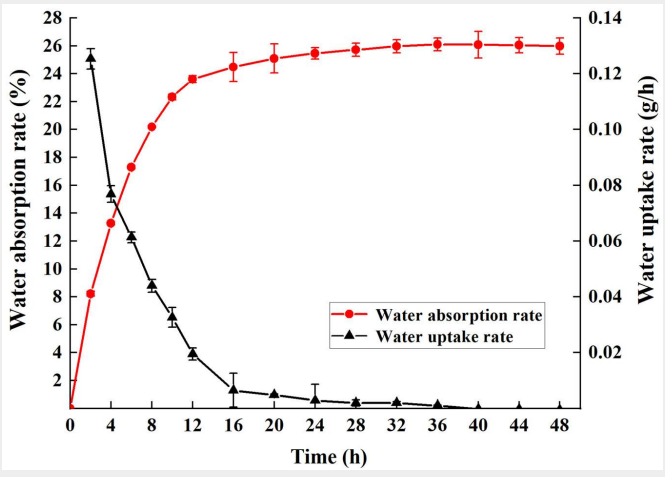

Fig. 1 - Water absorption dynamics of S.*tzumu* seeds.

Statistical analyses

All data was processed using the package Excel® 2019 (Microsoft, Redmond, WA, USA). Multiple comparisons were used to determine the differences between germination percentage, root length and seedling height of *B. campestris* seed by using the least significant difference (LSD – Bian et al. 2018) in SPSS® ver. 19.0 (IBM, Armonk, NY, USA), and drawn with Origin® 2018 (Northampton, MA, USA). The relationship between physiological parameters and germination percentage of *S. tzumu* seeds during wet sand storage was analyzed by R v. 4.0.3 (R Core Team 2020) using the packages "psych" (Revelle 2022) and "corrplot" (Wei & Simko 2021).

Results

Characteristics and germination test of S. tzumu seeds

The mature seeds were nearly circular with an average diameter of 5.00 mm, 1000-grain weight of 61.74 g and water content of 8.58%. Seed viability was 78.11%, which shows that the seeds have a high germination potential, and it is the premise of seed preservation (Tab. 1). The water absorption rate sharply increased in the earlier 12 h, slightly increased from 12 h to 24 h, and finally 26.09% of the initial weight of water was absorbed. The highest water uptake rate was 0.125 g h^{-1} in the initial 2 h. The intact seed stopped absorbing water after 24 h (Fig. 1).

The results showed that mature seeds could not germinate at different temperatures placed on the medium sand:leaf mold (1:3, v:v). Intact seed and embryo could not germinate on hormone-free medium, but 13.33% embryo germinated on medium with 1 g L^{-1} GA_3. In addition, the germination percentage of embryo without cotyledon on medium with hormone-free and 1 g L^{-1} GA_3 were 21.13% and 77.14%, respectively (Tab. 2). These results suggested that excised embryo produces normal seedling, and proved that embryo fully developed at maturity. Following our reasoning, however, the seed coat or cotyledon inhibited the germination of *S. tzumu* seeds.

Tab. 1 - Characters of *S. tzumu* seeds.

Seed characteristics	Measured value
Colour of seed coat	Black
Transversediameter	5.24 ± 0.16 mm
Longitudinal diameter	5.02 ± 0.14 mm
1.000-grain weight	61.74 ± 0.51 g
Water content	8.58 ± 0.09 %
Seed viability	78.11 ± 4.59%

Tab. 2 - Germination characteristics of *S.tzumu* seeds.

Medium	Seed type	GA_3 g·L^{-1}	Germination percentage (%)
MS	Intact seed	0	0
MS	Embryo	0	0
MS	Embryo without cotyledon	0	21.13 ± 1.25
MS	Intact seed	1.00	0
MS	Embryo	1.00	13.33 ± 0.58
MS	Embryo without cotyledon	1.00	77.14 ± 1.34

Inhibitory effect of S. tzumu seed extract

Compared to the control, the ethyl acetate extracts both in seed embryo and seed coat most significantly affected *B. campestris* seed germination percentage ($p < 0.01$). The effects of the water extract and ethyl acetate extract, methanol extract, and ether extract on germination percentage were significantly different ($p <$

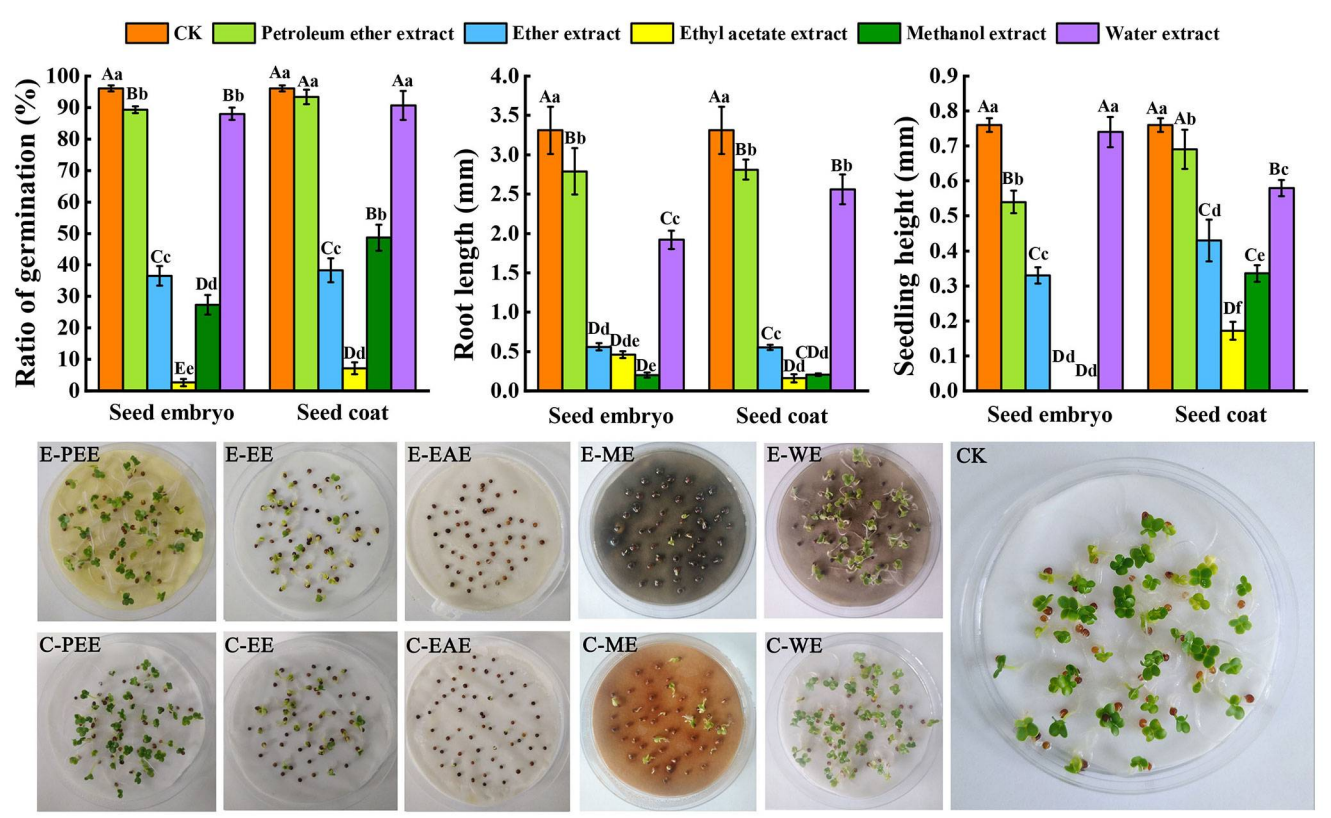

Fig. 2 - The effect of different solvent extracts on *B. campestris* seed germination. The different lowercase letters ($p < 0.05$) and uppercase letters ($p < 0.01$) indicate significant differences. The abbreviation E-PEE, E-EE, E-EAE, E-ME, and E-WE, respectively, stand for the petroleum ether extract, ether extract, ethyl acetate extract, methanol extract, water extract of seed embryo, whereas C-PEE, C-EE, C-EAE, C-ME, and C-WE stand for extracts of seed coat.

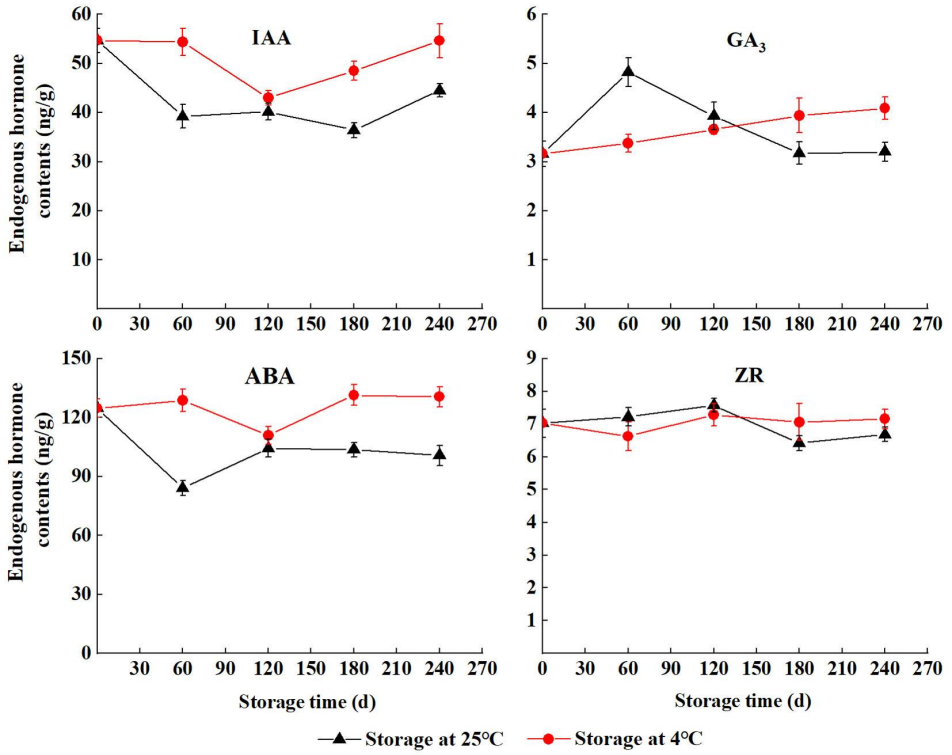

Fig. 3 - The endogenous hormone content of *S. tzumu* seeds during wet storage.

0.01). However, compared with the petroleum ether extract, the difference was not significant. In seed embryo and seed coat, the germination percentage of *B. campestris* using ethyl acetate extract was 2.64% and 7.18%, respectively. As compared to the control, the extracts of *S. tzumu* seeds significantly affected the growth of *B. campestris* roots ($p < 0.01$). The shortest roots of *B. campestris* were 0.2 mm (methanol extract of seed kernel added) and 0.16 mm (ethyl acetate extract of seed coat added). *B. campestris* seeds did not normally grow in the presence of ethyl acetate extract and methanol extract of the seed embryo and showed a significant difference in seedling height compared to the others ($p < 0.01$). In terms of the seed coat, the most significant effect on seeding height of *B. campestris* was observed using ethyl acetate extract, followed by methanol extract (Fig. 2).

Contents and ratio of seed endogenous hormones

The content of IAA in seeds initially decreased and then increased during the wet sand storage at 4 °C, and it was higher than that at 25 °C. The content of GA_3 storage at 25 °C rapidly increased in the first few days (S60 to S120), the highest value (4.82 ng g^{-1} FW) was found at S60 and then gradually decreased (S180 to S240). The content of GA_3 gradually increased with storage at 4 °C. The ABA profile was similar to the IAA. The lowest level of ABA at 4 °C and 25 °C were observed at S120 (110.93 ng g^{-1} FW) and S60 (84.04 ng g^{-1} FW), respectively. The levels of ZR storage at 25 °C were higher than that at 4 °C (S120 to S180), and the opposite was observed at S210 and S240 (Fig. 3).

The ratio of IAA/ABA initially decreased and then increased with storage at 4 °C, and the lowest was at S180. However, during storage at 25 °C, the ratio of IAA/ABA increased (S0 to S60), gradually decreased (S60 to S180), and then rapidly increased (S180 to S240). The level of GA_3/ABA rapidly increased with storage at 25 °C; the highest value was found at S60, and then sharply decreased (S60 to S240). The ratio of ZR/ABA decreased after an initial increase; moreover, it was always higher with storage at 25 °C than storage at 4 °C (Fig. 4).

Nutrients of S. tzumu seeds during storage

The content of soluble sugar, soluble protein, and free amino acid was higher at 4 °C than at 25 °C except for S240 (soluble protein). The level of soluble sugar gradually decreased during wet sand storage. The content of soluble protein showed the opposite trend at 4 °C and 25 °C with two peaks and two valleys at S60 and S180 separately. Fat content sharply decreased in the early stage of the storage (S0 to S60)

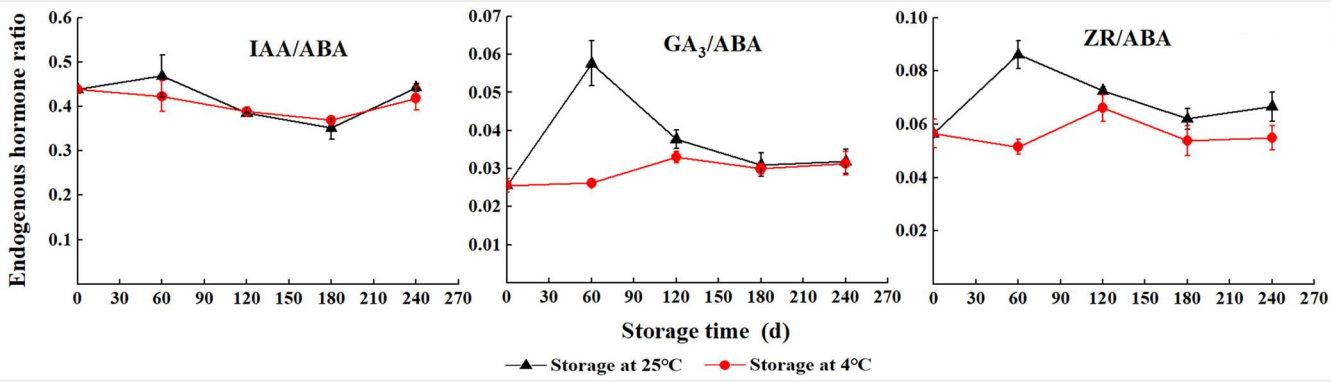

Fig. 4 - The endogenous hormone ratio of *S. tzumu* seeds during wet storage.

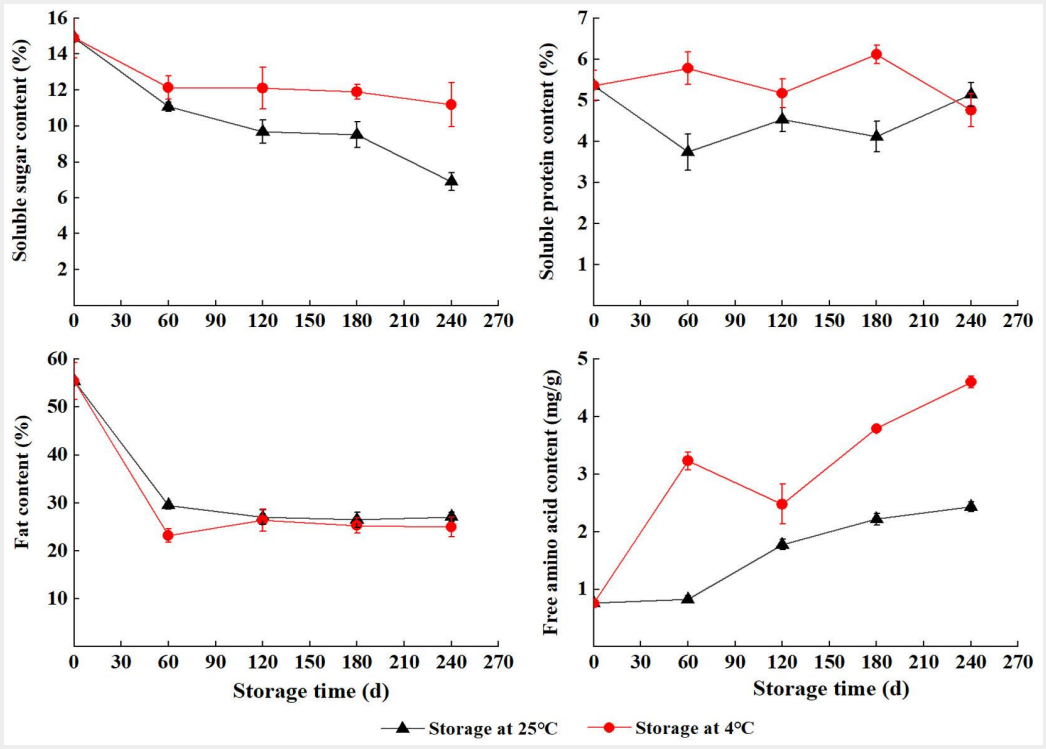

Fig. 5 - Nutrient content of *S. tzumu* seeds using different storage time.

and remained relatively constant during S120 to S240. The content of free amino acid first sharply increased (S0 to S60), slightly decreased (S60 to S120), and then sharply increased (S120 to S240) at 4 °C, and the highest was at S240 (4.6 mg g^{-1}). In contrast, the content of free amino acid steadily increased with storage at 25 °C (Fig. 5).

Germination percentage and effect of physiological parameters during storage

The germination percentage of *S. tzumu* seeds increased sharply with the extension of storage time. Germination was first observed at S60 (2.22%) and S120 (7.78%) under two temperature levels. After 8 months of wet sand storage, the germination percentage of *S. tzumu* seeds was 54.4% (stored at 4 °C) and 37.8% (stored at 25 °C – Fig. 6). Therefore, wet sand storage could accelerate seed dormancy release, and low temperature has a better curative effect. In seeds, significant positive correlations were found between germination percentage and GA_3 ($p < 0.05$), GA_3/ABA ($p < 0.05$), and free amino ($p < 0.01$), and negatively associated with soluble sugar ($p < 0.05$) storage at 4 °C. However, the germination percentage was negatively correlated to soluble sugar ($p < 0.01$) and positively correlated to free amino ($p < 0.01$) storage at 25 °C (Tab. 3).

Discussion

Seed characteristics and dormancy types

Seeds may possess various morphological and physiological characteristics that par-

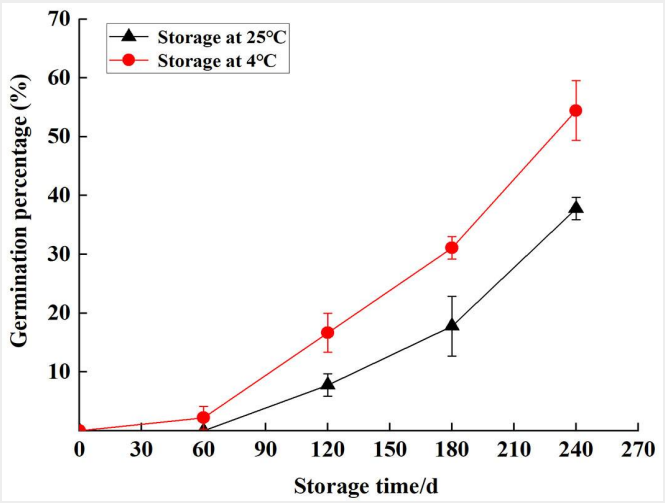

Fig. 6 - Germination percentage of *S. tzumu* seeds using different storage time.

Tab. 3 - Relationship between physiological parameters and germination percentage of *S. tzumu* seeds during storage.

Physiological parameters	Germination percentage of *S. tzumu* seeds			
	Storage at 4 °C		Storage at 25 °C	
	Correlation (*r*)	Probability values (*p*)	Correlation (*r*)	Probability values (*p*)
IAA	-0.028	0.921	-0.161	0.568
GA_3	0.855	<0.001	-0.487	0.066
ABA	0.286	0.301	-0.107	0.705
ZR	0.261	0.347	-0.499	0.058
IAA/ABA	-0.301	0.276	-0.113	0.689
GA_3/ABA	0.616	0.015	-0.331	0.228
ZR/ABA	-0.048	0.867	-0.215	0.442
Soluble sugar	-0.581	0.023	-0.787	<0.001
Soluble protein	-0.320	0.244	0.287	0.299
Fat	-0.488	0.065	-0.486	0.066
Free amino	0.802	<0.001	0.885	<0.001

tially represent the plant's response to selective pressure within the current environment during plant evolution (Tang et al. 2019). *S. tzumu* is always growing in sparse or dense forests (Wu et al. 2008), and previous research has suggested that ripe seeds are often scattered around the seed-bearer and only a few migrated with birds (Guan et al. 2006). In addition, it is difficult for seeds to reach distant areas through wind transport under natural conditions with a diameter of 5.00 mm and a 1000-grain weight of 61.74 g. A recent research has indicated that about 8.53%-27.22% of ripe fruits are without embryos (Chen et al. 2020), thus implying that the number of available seeds may be even lower. Water uptake is a fundamental requirement for intact seed germination (Guo et al. 2018) and is related to the structure of seed coat (Vu et al. 2014). In our study, a sufficient amount of water was absorbed in 12 hours to support the germination of seeds. The germination test, however, indicated that mature seeds cannot germinate, indicating that seeds are dormant at maturity. From an ecological perspective, the dormancy of *S. tzumu* seeds prevents germination and allows the seedlings to avoid severe winter conditions.

In this study, intact seed and embryo could not germinate on hormone-free medium. However, the germination percentage of the embryo without cotyledon on hormone-free medium was 21.13%. The results suggest that there may be a substance in the cotyledon or seed coat that significantly inhibits the germination of seeds. A bioassay test of *S. tzumu* seed extracts confirmed that extracts significantly inhibited radicle elongation and seedling height growth, which was also observed in other types of dormant seeds such as *Taxus yunnanensis* seeds (Bian et al. 2018) and *Grevillea* sp. seeds (Ma et al. 2015). Physical dormancy (PY) is caused by one or more water-impermeable layers of palisade cells in the seed coat, and the lack of imbibition in intact seeds serves as the hallmark for the identification of physical dormancy (Baskin & Baskin 2004). In this study, intact seeds rapidly imbibed water and increased in mass by 23.6% in the first 12 h. Therefore, we conclude that *S. tzumu* seeds are not physically dormant at maturity. The mature seeds cannot germinate, but the excised embryo produces normal seedling on MS medium with GA_3 (1 g L^{-1}). The germination percentage of embryo without cotyledon on medium with 1 g L^{-1} GA_3 was 77.14%, which proved that the embryo was fully developed at maturity. In addition, the treatment of cold stratification significantly improved the percentage of seed germination. According to the dormancy classification of Baskin & Baskin (2004), we can conclude that seeds of *S. tzumu* had intermediate physiological dormancy at maturity.

The balanced relationships among plant hormones play an important regulatory role in seed dormancy, including breaking seed dormancy and improving germination (Arteca & Arteca 2008). During the entire process of seed dormancy release, the contents of IAA decreased slightly and then increased. Generally, GA contributes to embryo development and increases seed germination (Lulsdorf & Ying 2013). In our study, the content of GA_3 slightly increased, which was similar to previous findings in *Paeonia lactiflora* (Li et al. 2020). Moreover, a positive correlation was found between the germination percentage of *S. tzumu* and GA_3 content ($p < 0.05$) with storage at 4 °C. The decrease in ABA content during long periods of cold stratification will trigger germination (Bian et al. 2013). In the seeds of *Arabidopsis* sp., physiological dormancy is associated with high levels of ABA and during dormancy breaking, ABA levels decrease (Staszak et al. 2019). However, the content of ABA was relatively stable except for S120 at 4 °C in this study, which suggested that the germination of *S. tzumu* seeds was not insensitive to the absolute concentration of ABA. Furthermore, a positive correlation was observed between germination percentage and GA_3/ABA ($p < 0.05$) that also proved that the balance between GA_3 and ABA significant affects dormancy release.

The quantitative and qualitative differences in seed nutrients may be related to the germination characteristics of species (Zhao et al. 2018), and different seed nutrients may have different roles during germination (Gu et al. 2016). Generally, the soluble sugar contents decreased with the extension of storage time. However, due to environmental stress and seed identity, the situation is more complicate. Lower total soluble sugar content has been associated with higher germination in *Jatropha curcas* seeds (Cui et al. 2014), but the opposite was observed during the storage of *Pseudolarix kaempferi* seeds (Han & Liao 2012). In this study, soluble sugar content decreased and was lower at 4 °C than at 25 °C, which may be due to weak respiration and less energy consumption storage at 4 °C. The dynamic changes in soluble protein and free amino acid play an important role in the process of seed internal material metabolism. During storage, the protein contents fluctuate with storage time and the amino acid contents gradually increase. This phenomenon indicates that protein synthesis and metabolism are active in seeds. However, the germination percentage is positively correlated to free amino and had no significant relationship with soluble protein. Our experiments showed that seeds have high fat content after harvest (55.4%) and rapidly decompose in the first two months. It is possible that seeds may retain vitality by decomposing a large amount of fat at the earlier stage of storage.

Conclusions

Overall, *S. tzumu* seeds are in intermediate physiological dormancy at maturity with high seed viability. The seeds normally absorb water and the physiological dormancy mainly result from the germination inhibitors in seed embryo and coat. Wet sand storage at 4 °C is an effective, low-cost method of breaking *S. tzumu* seed dormancy. Furthermore, the levels of GA_3, GA_3/ABA, soluble sugar, and free amino significantly affect the germination percentage of seeds during 4 °C storage. The results improve understanding of the *S. tzumu* seed dormancy, and provide the basis for seed preservation and reproduction for this specific plant species.

Acknowledgments

HC designed the study, conducted the experiment, and wrote the manuscript; JL, JJ and YL supervised the experiments at all stages and reviewed the manuscript; ZT supported the experimental material collection. All authors read and approved the final manuscript.

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