Phyton-
International Journal of
Experimental Botany

Tech Science Press

DOI: 10.32604/phyton.2023.029205

ARTICLE

Substrate, Hormone, Winnowing, and Stratification Influence the Seed Germination of *Ilex asprella* (Hook. et Arn.) Champ. ex Benth

Yuquan Huang[1,2], Fan Wei[1], Qing Ma[2], Yang Lin[1], Jinpeng Huang[2], Yanxia Zhu[1,*] and Danfeng Tang[1,*]

[1]Guangxi Key Laboratory of Medicinal Resources Protection and Genetic Improvement, Guangxi Botanical Garden of Medicinal Plants, Nanning, 530000, China

[2]Research and Development Center, China Resources Sanjiu Medical & Pharmaceutical Co., Ltd., Shenzhen, 518000, China

[*]Corresponding Authors: Yanxia Zhu. Email: zyx.1002@163.com; Danfeng Tang. Email: tdfmanuscript@163.com

Received: 07 February 2023 Accepted: 14 March 2023 Published: 31 May 2023

ABSTRACT

Ilex asprella (Hook. et Arn.) Champ. ex Benth is one of the most important traditional Chinese medicines in southern China. The seeds of *Ilex asprella* usually have extremely low germination due to their dormancy characteristics, which severely impacts the efficiency of seedling raising and increases labor costs. In this study, to improve the seed germination of *I. asprella*, the effects of germination substrate, hormone, winnowing, and stratification treatments on the seed germination of *I. asprella* were investigated. The results of the germination matrix showed that the highest germination percentage of 45.2% was achieved under the 20°C/10°C day/night temperature and vermiculite germination medium conditions. The results of hormone treatments revealed that 100–400 mg/L of gibberellin (GA) and 50–100 mg/L of salicylic acid (SA) were found to be effective in releasing the dormancy of *I. asprella* seeds. Moreover, winnowing could effectively eliminate unsaturated seeds and impurities, thus improving the seed germination of *I. asprella*. Furthermore, warm temperature (15°C) stratification could expand the temperature range of *I. asprella's* seed germination, which was beneficial for seed germination of *I. asprella* and for seed nursery at room temperature in production practice. The present study obtained a method to break dormancy and increase seed germination in *I. asprella*, thereby forming a groundwork for improving the efficiency of large-scale planting of *I. asprella*.

KEYWORDS

Ilex asprella; seed germination; substrate; winnowing; hormone; stratification

1 Introduction

Ilex asprella (Hook. et Arn.) Champ. ex Benth., commonly referred to as "Gangmei" in Chinese, belongs to the genus of holly in the Holly family and is native to Southern China [1,2]. The roots and leaves of *Ilex asprella* are primarily used as traditional medicine [3]. Its main active ingredients include polysaccharides, triterpenoid saponins, chlorogenic acids, phenols, and flavonoids [1,3,4], with a series of pharmacological and biological properties such as anti-inflammatory [5], antiviral [6,7], immunomodulatory [8], antitumor [9], antibacterial [10], antifungal [11], and hypolipidemic [12] activity. As a local medicinal herb in Lingnan regions, *I. asprella* is one of the main raw materials of many Chinese patent medicines and herbal teas [1,13].

Wild *I. asprella* is mainly distributed in the low mountainous areas of Guangdong, Guangxi, Fujian, and Jiangxi, with a slow growth rate naturally. In recent years, due to the destruction of the ecological environment and the large increase in demand for Chinese patent medicines, the supply of wild *I. asprella* has become scarce, leading to overexploitation of the resources and a huge disparity between supply and demand [2]. Therefore, it is imperative to carry out artificial cultivation of *I. asprella* in order to ensure sufficient market supply. Seed propagation is a common approach to artificially cultivate *I. asprella*. However, studies have shown that *I. asprella*'s seeds have a characteristic for dormancy, slow germination, and low germination rate [14–16].

Seed dormancy occurs when viable seeds fail to germinate under suitable conditions. It can be induced by several factors, such as immature seed embryos, immature seeds, seed coat impermeability, and the presence of germination-inhibiting substances [15,17]. To overcome seed dormancy, stratification and hormonal treatments are widely used. Stratification can be more effective in breaking seed dormancy by softening the seed coat and increasing permeability, as well as by enabling the seeds to undergo a series of physiological changes and maturation processes, and reducing or eliminating germination inhibitory substances [15]. Depending on the stratification temperatures, the stratification treatment can be categorized into low-temperature stratification (0°C–5°C), warm temperature stratification (15°C–25°C or 20°C–30°C), and variable temperature stratification [18]. Significant results have been achieved by using this stratification treatment on *I. argentina*, *I. ficifolia*, and *I. triflora* [19–21].

Compared with the stratification treatment, the application of exogenous plant hormones and growth regulators has emerged as a common method to promote seed germination. The commonly used exogenous hormones include gibberellins (GAs) and abscisic acid (ABA). As is well known, GAs are the primary hormones that facilitate the breakdown of seed dormancy by promoting the synthesis of auxin and cytokinin [17]. This also increases the activity of enzymes in the seed and promotes metabolic processes, resulting in the termination of seed dormancy and enhanced seed germination [22,23]. ABA is considered to play a major role in guiding seed maturation and sustaining seed dormancy under adverse environmental conditions until it is antagonized by GA and some environmental signals, thus allowing seed germination when the environmental conditions are favorable [24]. Additionally, in recent years, exogenous hormones such as salicylic acid (SA), melatonin (MT), and mepiquat chloride (MC/DPC) have received a lot of attention due to their role in breaking seed dormancy and promoting seed germination. SA, being a small phenolic molecule, has strong physiological effects under adverse plant conditions. It can improve seed germination under environmental stress, promote seedling growth and regulate various physiological and biochemical indices of stress resistance in plants [25–27]. Melatonin, a small indole-like molecule compound commonly found in plants, has been shown to improve seed germination and induce root production and growth. This is likely due to its involvement in the scavenging of reactive oxygen species and its vital role in physiological processes such as mature senescence, stress resistance, and immune regulation [28,29]. MC/DPC is a plant growth regulator with growth retarding functions that can increase seed vigor by promoting reductive and respiratory rates, thus increasing seed germination [30,31].

Winnowing grading is a method of removing impurities by using the difference in suspension speed between good seeds and unqualified seeds, and there is a significant difference between good seeds and unqualified seeds in terms of thousand-grain weight, so the purity and quality of seeds can be improved by winnowing [32]. The differences in nutrient content and physical structure of different substrates lead to differences in seed germination and growth, which are mainly reflected in the physiological indicators of seed germination rate, germination potential and seedling height [33]. Many studies have demonstrated that different substrates affect seed germination and that seed germination varies significantly under different substrate treatments [34,35].

During the harvesting, there are differences in the morphology of fruit and seeds of different batches of *I. asprella*, leading to fluctuating seed germination percentages that have a direct impact on the planting plan and planting scale of *I. asprella*. In addition, the seeds of *I. asprella* have a prolonged dormancy period, which results in a long period of seed germination and seedling growth. These two issues, namely the low and unstable seed germination and the extended seedling-raising period are the key constraints limiting the production and planting of *I. asprella*. Here, the effects of germination medium, hormone, winnowing, and stratification treatments were studied to improve the seed germination of *I. asprella* and shorten the seedling raising time. This study aimed to develop an effective method for the germination of *I. asprella* seeds and provide a scientific reference for improving the efficiency of large-scale planting of *I. asprella*.

2 Materials and Methods

2.1 Seed Preparation

On June 26th, 2022, the mature seeds of 4-year-old *I. asprella* plants were collected from the Maodaoyuan *I. asprella* planting base in Hezhou, Guangxi, China. The average weight of 100 seeds was 0.513 g, with a water content of 7.73%.

2.2 Germination Medium Screening Test

A germination box measuring 12 cm × 6 cm × 6 cm was filled with moist fine sand (particle size 0.1–0.2 mm, 200 g river sand + 40 g purified water), moist perlite (particle size 3–5 mm, 15 g perlite + 60 g purified water), moist vermiculite (particle size 1–2 mm, 30 g vermiculite + 80g purified water), and moist filter paper (2 layers of filter paper +10 ml purified water) to represent the germination bed. Subsequently, 200 seeds were evenly placed throughout the germination box. Then the germination box was placed in a light incubator for germination culture with a photoperiod of 12 h light/day, and a constant temperature of 10°C, 15°C, 20°C, 25°C, and 30°C, as well as a day/night variable temperature of 20°C/10°C, 25°C/15°C, and 30°C/20°C, respectively. Each treatment was repeated three times. The germination percentage was calculated on the 100th day after sowing.

2.3 Germination Progress

To further observe the germination progress of *I. asprella* seeds, the germination percentage was recorded over time under the optimum germination medium (vermiculite) and temperature (20°C/10°C day/night).

2.4 Hormonal Treatment Experiment

For the hormonal treatment experiment, 3 g of dried seeds were soaked in gibberellin (50, 100, 200, 400 mg/L), melatonin (1, 5, 10, 20, 40 mg/L), abscisic acid (1, 5, 10, 20, 40 mg/L), salicylic acid (1, 10, 20, 50, 100 mg/L), and mepiquat chloride (1, 5, 10, 20, 40 mg/L) solutions for 10 h, with the distilled water immersion as the control, then rinsed 3~5 times with clean water. Subsequently, the seeds were evenly placed on a moist vermiculite germination bed and placed in a light incubator for germination (photoperiod of 12/12 h light/dark, the temperature of 20°C/10°C day/night). Each treatment was repeated four times and each repetition contained 100 seeds. After 90 days, the germination percentage was calculated.

2.5 Seed Winnowing Test

3 g of dried seeds were placed in a fanning mill (model CFY-4, Zhejiang Topyunnong Technology Co., Ltd., China) with a wind flow of 2.5 m^3/min for 120 s. The seeds (wind-selected and control) were placed on the wet vermiculite germination bed and then were laid in the light incubator for germination (the photoperiod of 12 h light/day, the germination temperatures of 20°C/10°C day/night, 10°C, and 15°C).

After 90 days, we calculated the germination percentage. Each treatment was replicated four times and each repeat contained 100 seeds.

2.6 Stratification Test

The seeds that had been selected by the wind were mixed with the wet perlite (the weight ratio of perlite: water is 1:2) in a volume ratio of 1:1 and incubated for 2 months at a constant temperature of 5°C, 15°C, and 25°C for stratification treatment. Then, these seeds were placed on the wet vermiculite germination bed and germinated in the light incubator (the photoperiod was 12 h light/d, and the temperature was set to be a series of the constant temperatures of 10°C, 15°C, 20°C, 25°C, and 30°C and a series of day/night variable temperatures of 20°C/10°C, 25°C/15°C and 30°C/20°C, respectively). After 80 days, we calculated the germination percentage. The dry seeds without stratification were set as the control. Each treatment was repeated three times and each repeat contained 100 seeds.

2.7 Germination Standard and Calculation

When the radicle emerged through the seed coat and its length exceeded 2 mm, the seed was deemed to have germinated. The germination ended when the number of germinated seeds did not increase for 7 consecutive days.

Germination percentage = Number of germinated seeds/number of tested seeds × 100%.

2.8 Statistical Analysis

SPSS 23.0 software was used for statistical analysis and the data means were analyzed using the Duncan test for statistical significance ($p < 0.05$). Each test was analyzed independently and the test for homogeneity of variance was performed before ANOVA. WPS software was employed for graphing.

3 Results

3.1 Germination Medium Screening Test

Table 1 showed that 20°C/10°C had the highest germination percentage among the different germination temperatures tested, followed by 10°C and 15°C, conversely, the seeds of *I. asprella* barely germinated at the other temperatures. At 20°C/10°C, the germination percentage of vermiculite treatment was significantly higher than that of the fine sand, perlite, and filter paper treatments and increased by 13.4%, 19.9%, and 411.6%, respectively. Under a constant temperature of 10°C, the germination percentage of *I. asprella* seeds varied as follows: vermiculite (35%) > fine sand (30.8%) > perlite (16.3%) > filter paper (4.3%), while at a constant temperature of 15°C, the order of the germination rate of *I. asprella* seeds was fine sand (17.7%) > vermiculite (4.5%) > perlite (3.2%) > filter paper (2.7%). In a word, the highest germination percentage (45.2%) was obtained under the treatment of 20°C/10°C day/night temperature and vermiculite germination medium conditions, which was more conducive to the seed germination of *I. asprella*. This could be because the vermiculite germination medium was more effective in providing insulation and moisture for the seeds of *I. asprella*, thus making the germination of the seeds more favorable.

3.2 Germination Progress of I. asprella Seeds

As could be observed from Fig. 1, the germination of *I. asprella* seeds commenced on the 50^{th} day, with subsequent germination percentages calculated every 10 days from the 60^{th} to the 100^{th} day being 3.8%, 20.2%, 32.3%, 45.2%, and 47.7%, respectively. Particularly, during the time intervals of 60^{th}–70^{th}, 70^{th}–80^{th}, and 80^{th}–90^{th}, the germination percentage of *I. asprella* seeds had a growth rate greater than 10%, with respective figures of 16.4%, 12.1%, and 14.9%. However, from the 90^{th} day to the 100^{th} day, the germination percentage increased by a rate of 5.5%. This indicated that the seed germination percentage of *I. asprella* tended to be flat.

Table 1: Effect of different germination mediums on the germination percentage of *I. asprella* seeds. Different lowercase letters in the same column and different capital letters in the same line indicate significant differences, $p < 0.05$

Germination temperature (°C)	Fine sand	Perlite	Vermiculite	Filter paper
10	30.83 ± 2.25bB	16.33 ± 1.89bC	35.00 ± 2.65bA	4.33 ± 1.26bD
20/10 (day/night)	39.83 ± 3.014aB	37.67 ± 1.26aB	45.17 ± 2.25aA	8.83 ± 4.01aC
15	17.67 ± 2.75cA	3.17 ± 0.29cB	4.50 ± 1.50cB	2.67 ± 1.53cB
25/15 (day/night)	0.33 ± 0.29dA	0.83 ± 0.76dA	0.83 ± 0.58dA	0.33 ± 0.29dA
20	0.17 ± 0.29dA	0.00 ± 0.00dA	0.00 ± 0.00dA	0.00 ± 0.00dA
30/20 (day/night)	0.17 ± 0.29dA	0.00 ± 0.00dA	0.83 ± 1.04dA	0.00 ± 0.00dA
25	0.17 ± 0.29dA	0.00 ± 0.00dA	0.00 ± 0.00dA	0.00 ± 0.00dA
30	0.00 ± 0.00dA	0.00 ± 0.00dA	0.00 ± 0.00dA	0.00 ± 0.00dA

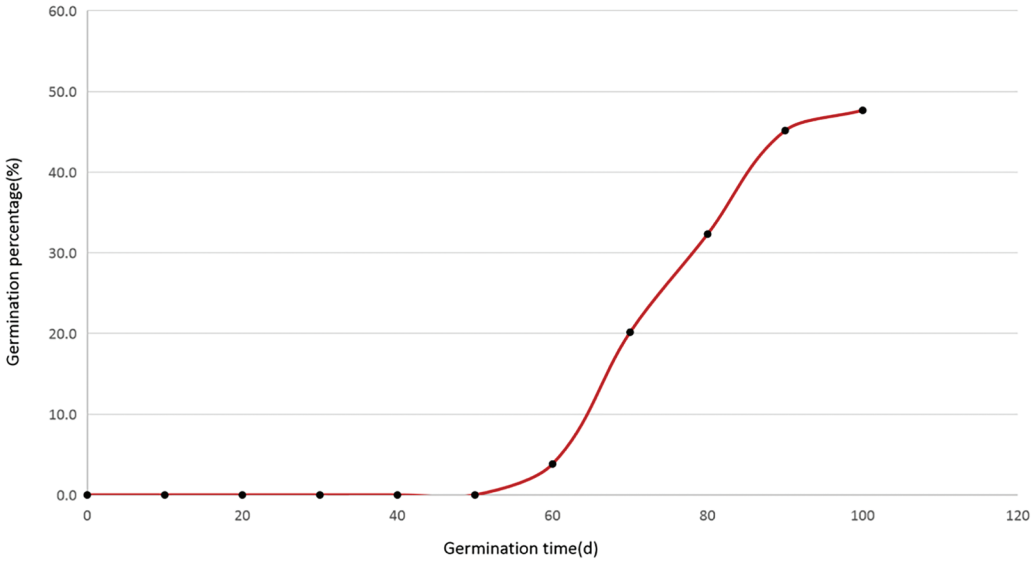

Figure 1: Germination progress of *I. asprella* seeds under 20°C/10°C day/night and vermiculite condition

3.3 Hormonal Treatment Experiment

Different hormones had different effects on the germination percentage of *I. asprella* seeds. The germination percentage of *I. asprella* seeds was significantly affected by the concentration of GA ($p < 0.05$; Fig. 2A). With the increase in GA concentration, the germination percentage of *I. asprella* seeds increased first and then became flat. The germination percentage of the seeds treated with different concentrations of GA decreased in the order of 400 mg/L GA (60.13%) > 100 mg/L GA (58.25%) > 200 mg/L GA (56.88%) > 50 mg/L GA (52.5%) > CK (42.88%).

No statistically significant difference in seed germination was observed at 1, 10, and 20 mg/L SA compared to the control ($p > 0.05$). However, a significant increase in seed germination percentage was observed at 50 mg/L SA (47.50%) when compared to the control (42.88%). The treatment with 100 mg/L SA had the most positive effect on germination, resulting in a germination percentage of 54.33%, which

was 14.38%, 22.78%, 16.84%, 25.39%, and 26.70% higher than that of the treatments with 50, 20, 10, 1 mg/L SA and the control, respectively (Fig. 2B). It was indicated that the high concentration of SA had a promoting effect on the seed germination of *I. asprella*. On the contrary, the MT, ABA, and DPC treatments dramatically reduced the seed germination percentage of *I. asprella* ($p < 0.05$), compared with the control (Figs. 2C–2E). Conversely, with the increase in the concentration of MT, ABA, and DPC, the germination percentage of *I. asprella* seeds tended to decrease, indicating that MT, ABA, and DPC had an inhibitory effect on the germination of *I. asprella* seeds.

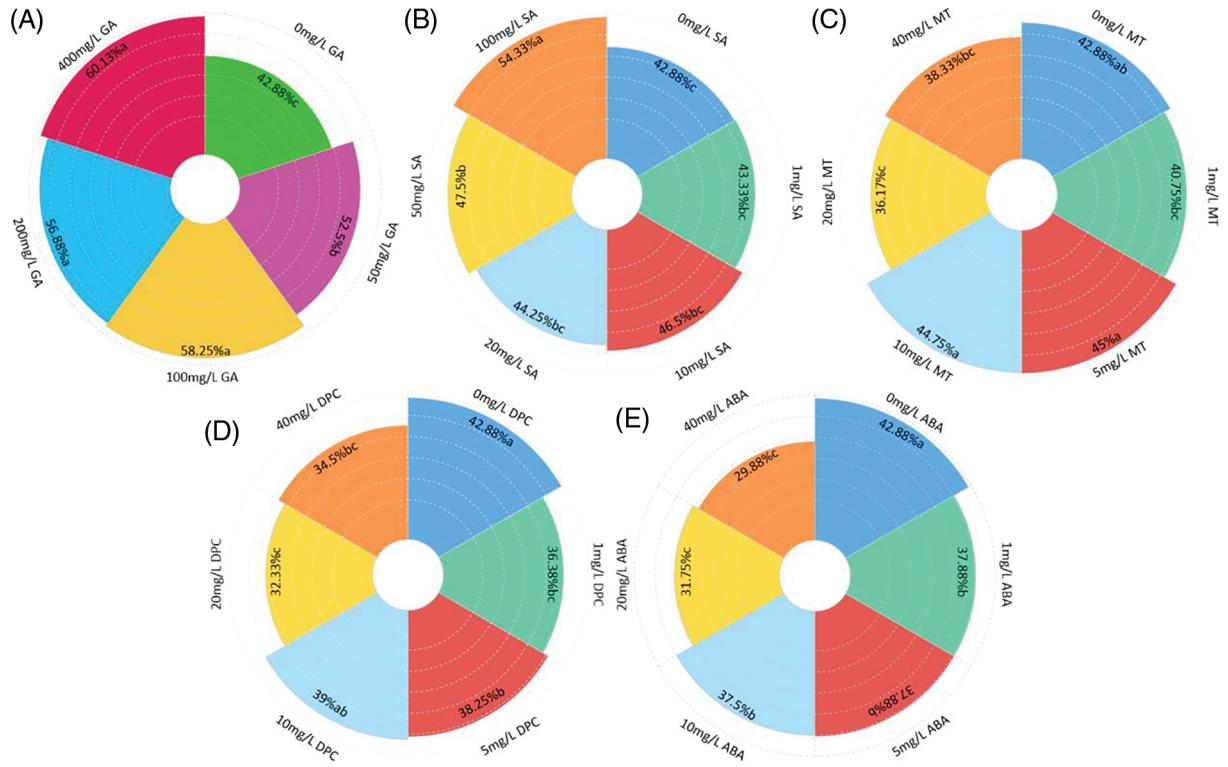

Figure 2: Effects of hormone soaking on the seed germination of *I. asprella*. (A) The effects of different GA concentrations on seed germination. (B) The effects of different SA concentrations on seed germination. (C) The effects of different MT concentrations on seed germination. (D) The effects of different DPC concentrations on seed germination. (E) The effects of different ABA concentrations on seed germination

3.4 Seed Winnowing Test

There was a significant difference ($p < 0.05$) in the 100-grain weight of the winnowing seeds of *I. asprella* compared with the control (Fig. 3A). The winnowing seeds had a 100-grain weight of 0.62 g, which increased by 21.57% in comparison with the control. The winnowing treatment also significantly affected the seed germination percentage of *I. asprella* seeds ($p < 0.05$) (Fig. 3B). At 20°C/10°C, 15°C, and 10°C, the germination percentage of the winnowing seeds were 68.3%, 51.3%, and 41.3%, respectively. The highest germination percentage was 68.3% at 20°C/10°C, representing an increase of 23% higher than that of the seeds without winnowing.

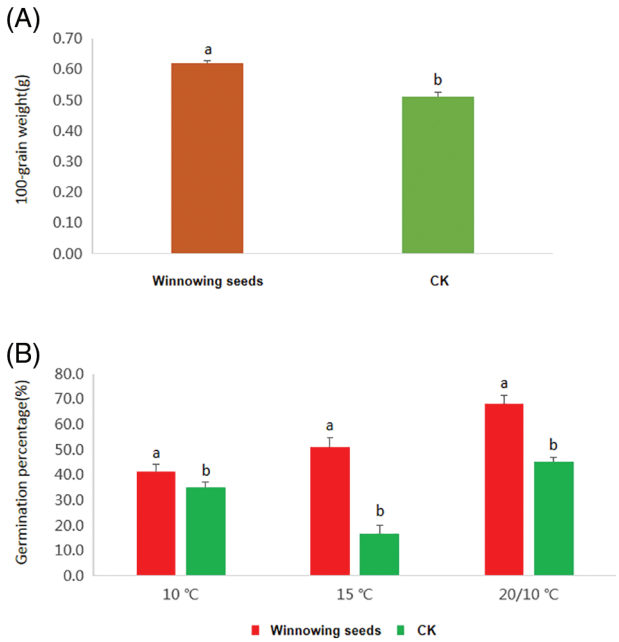

Figure 3: Effects of winnowing on the seed germination of *I. asprella*. (A) The comparison of 100-grain weight between the winnowing seeds and the control. (B) The comparison of germination percentage between the winnowing seeds and the control under different germination temperatures. CK = No stratification

3.5 Stratification Test

To observe the effects of stratification temperature on the seed germination of *I. asprella*, the changing trend of the germination of the wind-selected seeds in perlite was monitored after stratification at 5°C, 15°C, and 25°C for 2 months. As was evident in Table 2, the stratification treatment not only improved the seed germination percentage but also expanded the temperature range in which *I. asprella* seeds could germinate. The seeds without stratification treatment hardly germinated under higher temperature conditions (25°C/15°C, 20°C, 30°C/20°C, 25°C, and 30°C). The highest germination percentage without stratification was 68.3% under the 20°C/10°C condition. It was indicated that low temperature was favorable for the seed germination of *I. asprella*. In particular, after the 15°C stratification treatment, the seeds germinated at different germination temperatures, and the germination percentage was higher than 20%. After the stratification treatment at 15°C, the highest germination percentage was 73% under the germination condition of 20°C/10°C, which was 4.7% higher than that of the control ($p > 0.05$). It was inferred that the 15°C stratification treatment could significantly increase the seed germination and expand the temperature range of seed germination.

Table 2: Effect of stratification temperature on the seed germination of *I. asprella*. Different lowercase letters in the same column and different capital letters in the same line indicated significant differences, $p < 0.05$

Germination temperature (°C)	Stratification temperature (°C)			
	5	15	25	CK (No stratification)
10	7.00 ± 3.46cC	33.33 ± 4.04dB	6.67 ± 2.52bC	41.25 ± 2.87cA
20/10 (day/night)	19.67 ± 3.21abB	73.00 ± 3.00aA	15.00 ± 2.00aB	68.25 ± 3.30aA
15	2.00 ± 1.00dC	48.00 ± 4.00cA	8.33 ± 3.06bB	51.25 ± 3.69bA
25/15 (day/night)	21.67 ± 1.15aB	53.67 ± 4.73bcA	0.00 ± 0.00cC	0.00 ± 0.00dC

(Continued)

Table 2 (continued)

Germination temperature (°C)	Stratification temperature (°C)			
	5	15	25	CK (No stratification)
20	16.67 ± 1.53bB	55.67 ± 3.51bA	13.67 ± 1.53aB	0.00 ± 0.00dC
30/20 (day/night)	1.00 ± 0.00dB	27.33 ± 0.58efA	0.00 ± 0.00cB	0.00 ± 0.00dB
25	1.00 ± 1.00dB	29.33 ± 1.15deA	0.00 ± 0.00cB	0.00 ± 0.00dB
30	0.33 ± 0.58dB	22.33 ± 3.51fA	0.00 ± 0.00cB	0.00 ± 0.00dB

4 Discussion

Seed formation is closely related to the plant's genetic features and environmental conditions, and it is generally believed that larger seeds are considered to facilitate the reproduction and development of the species [36]. The winnowing treatment could remove unsaturated seeds and impurities and improves the clarity and purity of the seeds. Previous studies have shown that the thousand-grain weight of *I. asprella* seeds is positively correlated with germination percentage [37]. In the present study, after winnowing treatment, the 100-grain weight of the seeds was heavier and the seed germination percentage observed was significantly higher than that of the control seeds (Fig. 3), indicating that the results were consistent with the above findings [37].

Substrate, a light material, is an effective substitute for soil, as it provides plants with the necessary nutrients and growth environment. It is abundant in key nutrients such as nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulfur (S), and other essential nutrients to support plant growth and development, thus reducing the need for fertilizers. Common substrates include vermiculite, perlite, fine sand, etc. [38]. In this study, at the optimum germination temperature (20°C/10°C), vermiculite had the best treatment effects (Table 1). It might be because vermiculite harbored the characteristics of improving the soil aggregate structure and storing water and moisture, resulting in better moisturizing and fertilizing, which was more conducive to the seed germination of *I. asprella* [39].

The internationally acceptable hierarchical system of classification for seed dormancy includes five classes of dormancy: physiological dormancy (PD), morphological dormancy (MD), morphophysiological dormancy (MPD), physical dormancy (PY), and combinational dormancy (PY+PD) [17]. Based on cold and/ or warm stratification requirements for germination, temperature requirements for embryo growth, and response to GA3, MPD can be classified into nine types: non-deep simple, intermediate simple, deep simple, deep simple epicotyl, non-deep simple epicotyl, deep simple double, non-deep complex, intermediate complex, and deep complex MPD [40]. Among them, the feature of non-deep simple is that the seed embryo grows and develops under warm stratification (>15°C). Additionally, GA can shorten the stratification time and reduce the depth of dormancy [40]. In this study, the effect of warm temperature stratification at 15°C was significantly better than that at 5°C and 25°C (Table 2), and the concentration of GA at 100–400 mg/L could significantly improve the seed germination of *I. asprella* (Fig. 2). Therefore, it was indicated that the seeds of *I. asprella* might belong to the non-deep simple MPD type. Moreover, only the high concentration of SA significantly increased the germination of *I. asprella* seeds (Fig. 2). This indicated that the most suitable concentration of SA for the germination of *I. asprella* seeds needed to be further studied. In addition, with the increase of the concentrations of MT, ABA, and DPC, the seed germination of *I. asprella* tended to decrease, suggesting that MT, ABA, and DPC had an inhibitory effect on the germination of *I. asprella* seeds.

In the Lingnan region, the ideal climatic conditions for *I. asprella* growth are an annual average temperature of 20°C–28°C, with an average of 24°C–31°C in summer and 13°C–25°C during the spring

and winter. Even if the seeds are collected and sown immediately in July of that year, they will not germinate until the next spring [16]. In addition to the dormancy characteristics of the seeds, the reasons might also be related to the high temperature in summer, which was not suitable for the germination of the seeds. In this study, the seeds without stratification (CK) were capable of germinating under constant temperatures of 10°C and 15°C, as well as altering temperature of 20°C/10°C. This also confirmed that the germination of the seeds of *I. asprella* prefered the seasons and temperatures of autumn, winter, and spring in South China.

Studies have shown that seed germination in many species requires temperature fluctuations [41–43]. In the present study, regardless of whether the seeds of *I. asprella* were treated by stratification or not, the germination of *I. asprella* seeds under 20/10 variable temperature conditions was significantly higher than that under other constant temperature conditions. *I. asprella* is a deciduous shrub, which often grows in roadside bushes or broad-leaved forests in the valley at an altitude of 400–1000 m. The suitable temperature for growth is 16°C–25°C, and it especially prefers semi-shady and semi-sunny environments [2]. Therefore, it was inferred that the lack of fluctuation of ambient temperature might also be one of the important reasons limiting the germination of *I. asprella* seeds.

Stratification treatment is a useful physical method to release plant seed dormancy, which can break seed dormancy and improve seed germination. In this study, the seeds from low-temperature stratification (5°C) and high-temperature stratification (25°C) had a low germination percentage (<22%) at all germination temperatures, while the seeds from warm-temperature stratification (15°C) at all germination temperatures harbored higher germination (Table 2). Although the germination percentage of the seeds from warm temperature stratification was not significantly different from that of the control at the germination temperatures of 20°C/10°C, the temperature range of seed germination of *I. asprella* was greatly expanded after warm temperature stratification. It was indicated that the low germination temperature and warm temperature stratification conditions were favorable for seed germination of *I. asprella* and for seed nursery at room temperature in production practice.

5 Conclusion

In this study, vermiculite was found to be the most suitable germination medium, and the optimum germination temperature was 20°C/10°C day/night for the seeds of *I. asprella*. 100–400 mg/L gibberellin (GA) and 50–100 mg/L salicylic acid (SA) were effective methods to break the dormancy of *I. asprella* seeds. In addition, winnowing was found to be an effective method for removing unsaturated seeds and impurities, thus improving seed germination. A warm temperature (15°C) stratification was also shown to expand the temperature range of seed germination of *I. asprella*. Overall, the present study provided the conditions where a high germination percentage of *I. asprella* seeds could be obtained.

Funding Statement: This study was supported by the Fund Projects of the Central Government in Guidance of Local Science and Technology Development (GuiKeZY22096020), Natural Science Foundation of Guangxi (2019GXNSFBA245073), National Natural Science Foundation of China (82260750, 82260749), and Cooperative Project of Guangxi Botanical Garden of Medicinal Plants with China Resources Sanjiu Medical & Pharmaceutical Co., Ltd. (202112-1).

Author Contributions: Conceptualization: D.F.T. and Y. X. Z.; methodology: Y.H., Q.M., Y.L. and J.H.; formal analysis: F.W.; writing-review and editing: D.F.T. All authors reviewed the results and approved the final version of the manuscript.

Availability of Data and Materials: All data generated or analyzed during this study are included in this article.

Ethics Approval: In this study, the collection of plant materials complies with relevant institutional, national, and international guidelines and legislation.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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