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Study on the Dormancy Characteristics of Chinese Pistache (*Pistacia chinensis* Bunge) Seeds

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Abstract: Chinese pistache tree (*Pistacia chinensis* Bunge, Anacardiaceae) is an important oil, ornamental and afforestation tree species. Seeds of this species are known due to their erratic germination, so it is urgent to determine the causes of such dormancy. To do so, we tested water permeability, respiration rate, mechanical constraint and germination inhibitors. The results indicated that the testa was water-permeable, the raphe and hilum as the areas that contributed most to imbibition. Removal of the testa increased the imbibition rate and improved the oxygen availability to the embryo. Furthermore, the analysis of the extracts showed that the endotesta and embryo may contain inhibitors that negatively affect germination and seedling growth. The widely targeted metabolomics revealed that phenolic acids and organic acids may be the potential inhibitors. Seed scarification, exogenous gibberellin (GA_3) and cold stratification greatly increased the germination, but seed scarification (removal of the exotesta and 1/2 cotyledon) was the most effective strategy, since it increased the germination up to 89% after 5 days of incubation, a rate significantly higher regarding the other strategies, which barely reached 60% in 20 days. In conclusion, there seems to exist both a mechanical dormancy due to the testa and a physiological inhibition due to the embryo.

Keywords: Chinese pistache; imbibition; respiration; inhibitors; scarification

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1. Introduction

Chinese pistache (*Pistacia chinensis* Bunge) is a deciduous woody oil tree species belonging to the Anacardiaceae. It is native to China and covers a wide area. The distribution of Chinese pistache includes two major centers, in Central Asia and Southern China, with some patches in Southern Japan and the Indonesian Islands [1]. The Chinese pistache tree has a beautiful shape, red leaves in early spring and dark-red or orange leaves in autumn, and high ornamental value [2]. It can grow in slightly acidic, neutral, and slightly alkaline soils, with strong adaptability and stress resistance. In addition, the Chinese pistache tree is often regarded as an important afforestation tree species, timber tree species, and medicinal plant [3–6]. The seeds of Chinese pistache have a high oil content (42.26%); therefore, it is listed as one of the seven woody oil tree species in China by the National Energy Administration and National Forestry and Grassland Administration. It is of great significance to the improvement of the total amount of biomass energy, especially nongrain biomass energy, in China [7,8]. It has been reported that all *Pistacia* species can be used as rootstocks for pistachios (*Pistacia vera* L.) in horticulture [9]. In China, there is only a few pistachio cultivations, and the expensive pistachios have relied on foreign imports in recent years. The local abundant Chinese pistache resources are excellent rootstocks for pistachios grafting, with a survival rate of over 85%. The sowing method has usually been adopted in Chinese pistache seedling production. However, seeds have deep dormancy properties that make germination difficult, and thus, proper treatments are required to break their dormancy [10,11].

Seed dormancy is an adaptive plant characteristic that allows seeds to disperse and spread under different time and space conditions to improve the survival rate of plants.

Seeds only germinate when the external environmental conditions are appropriate [12]. Female parents play an important role in the dynamic regulation of the seed dormancy of offspring [13]. Fedi et al. (2017) identified a large number of seed coat mutants, emphasizing the significant function of the female parent tissue in the regulation of seed dormancy [14]. Moreover, the environmental conditions in which the female parent remains have a great impact on seed dormancy, and the seed germination time is strongly selective [15]. Testa and endosperm are the tissues surrounding the embryo. They may influence water imbibition and gas exchange, hinder radicle protrusion, prevent the exudation of inhibitors from the embryo or provide inhibitors to the embryo, and affect light penetration [16]. Slator et al. (2013) found that the valve structure was an important factor leading to the poor germination of mechanically dormant teak (*Tectona grandis* L.f.) seeds [17]. Mechanical dormancy was imposed by the hard pericarp of *Lepidium didymum* L. seeds, since the pericarp blocked the full-phase II water uptake; however, the dormancy could be released through common fungal activity to weaken the zones by degrading nonlignified pericarp cells [18]. For physically dormant black locust (*Robinia pseudoacacia* L.) seeds, the strophiole is a key site for water absorption into the embryo under natural conditions [19]. In addition, Mexican yew (*Taxus globosa* Schltdl.) seeds have the dormancy properties caused by immature embryos and physiological inhibitors (such as abscisic acid, ABA) in embryos, and warm stratification combined with low-temperature stratification and the application of gibberellin (GA) are needed to promote seed germination [20].

The seeds of *Pistacia* species are surrounded by a hard sclerotic exotesta that makes germination difficult. Scarification and cold stratification were found to improve the seed germination in *Pistacia* spp. [21]. The germination of pistachio seeds is generally low under ordinary conditions. Cold stratification can enhance the germination capacity of pistachio kernels [9]. The higher germination of *Pistacia khinjuk* Stocks and *Pistacia atlantica* Desf. was obtained by scarification treatment [21,22]. Rich phenolic compounds in seeds and kernels of Atlas pistachio (*Pistacia atlantica* Desf.) limit seed germination [23]. The sowing method is usually adopted in the seedling production of Chinese pistache in China. However, the seeds have deep dormancy characteristics with erratic germination, and low-temperature (4 °C) stratification is usually adopted to break their dormancy [10,11]. Zhao et al. (2004) applied low-temperature sand stratification for 100 days to Chinese pistache seeds to achieve the highest germination of 16.2% [24]. Additionally, after Chinese pistache seeds were stored in low-temperature sand for 3 months, the average germination was 20.25% in the laboratory and only 14.5% in the nursery [25]. However, Cole (1994) found that the germination rate of Chinese pistache seeds could reach more than 90% with cold stratification, which may be attributed to the different habitats, climates, or seed lots [26].

Therefore, we speculated that the testa-induced mechanical barrier and seeds/embryo-induced physiological inhibition may lead to the dormancy property of Chinese pistache seeds. In this study, the water permeability, respiration, mechanical constraint, and germination inhibitors of Chinese pistache seeds were tested to explore their dormancy characteristics to understand the dormancy reason, improve the seed germination rate, and increase seedling production.

2. Materials and Methods

2.1. Seed Materials

Fruits of Chinese pistache were collected from a wild scattered forest in Guxin Village, Guxin Town, Shexian County, Handan City, Hebei Province, on 18 September 2020, when the fruit changed from red or orange-red to copper green. The pericarp should be removed from all Chinese pistache seeds immediately after harvest [26]. Thus, the pericarp of the collected fruits was rubbed off in a timely manner (Figure 1), the fruits were rinsed with running water and air-dried at room temperature of 25 °C, and the obtained seeds with a moisture content of 8.38% were stored in a 4 °C refrigerator for later use. The seeds had an average size with a length of 5.10 mm, a width of 4.34 mm, and a thickness of 3.33 mm,

with a 1000-grain weight of 36.81 g. In addition, the percentage of seed vigor was 85% by the TTC (triphenyl tetrazolium chloride) staining method [27]. Commercial cabbage seeds (with a germination rate higher than 96%) were used to evaluate the inhibitory effect of the extract Chinese pistache seeds.

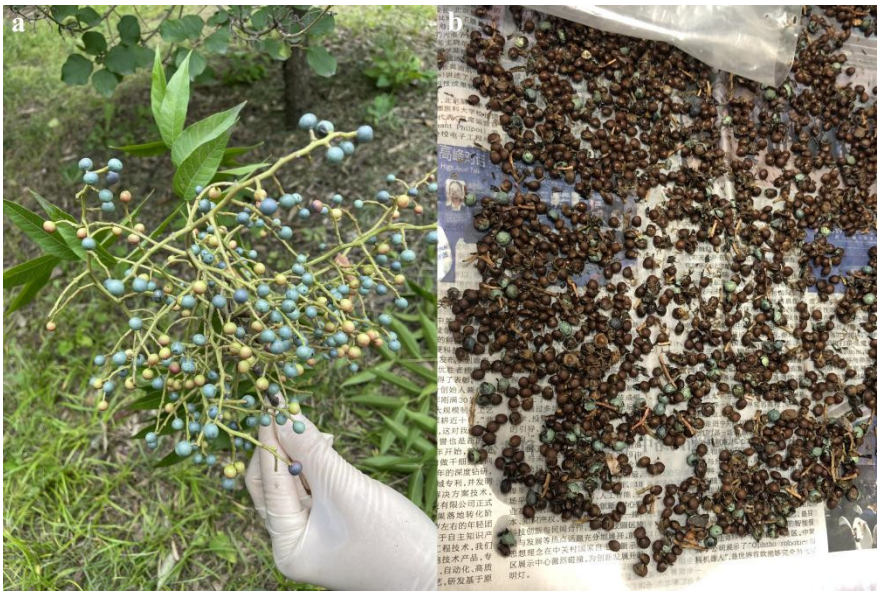


Figure 1. Harvested Chinese pistache seeds with the pericarp (**a**) and without the pericarp (**b**).

2.2. Water Permeability of Chinese Pistache Seeds

2.2.1. Water Absorption Rate

Healthy seeds of Chinese pistache of the same size were weighed, put into a beaker, and soaked in distilled water. Then, the beaker was placed in an incubator with a photoperiod of 16-h light and 8-h dark at 25 ± 1 °C, relative humidity of 65%, and light intensity of 340 mol m^{-2} s^{-1} for 72 h. According to Zhao et al. (2004), Chinese pistache seeds are saturated after soaking in water for 72 h [24]. The seeds were removed regularly (every 4 h during the daytime and 12 h at night), and the surface water was blotted off with filter paper. Then, the seeds were quickly weighed with an electronic balance at room temperature until the weight did not change. A completely randomized design was adopted, and there were three replicates and 50 seeds per replicate in this test. The water content was calculated based on the following formula:

$$\text{Water content (\%)} = [(W_t - W_0)/W_0] \times 100\% \quad (1)$$

where W_t is the weight at one time, and W_0 is the initial weight.

2.2.2. Water Imbibition under Different Seed Coat Weakening Treatments

The imbibition test was conducted to determine the mass increase of the following treated seeds:

a. intact dry seeds (DS): referring to dormant seeds, with an initial moisture content of 8.38%.
b. imbibed seeds (BS): referring to those imbibing for 72 h and becoming saturated, with an initial moisture content of 37.2%.
c. cracked seeds (CS): with a gap size of 0.2 mm along the raphe and an initial moisture content of 38.9%.
d. exotesta-removed seeds (EXS): being obtained by peeling off the exotesta of BS with a scalpel, with an initial moisture content of 37.2%. BS were used, because a small

fissure along the raphe appeared when the seeds were wiped dry. Then, it was easy to remove the exotesta, and the embryo was intact and not easy to crush.

e. endotesta-removed seeds (ENS): being obtained by peeling off the endotesta of EXS, with an initial moisture content of 37.2%.

A completely randomized design was adopted in this test. Three replicates of 60 seeds each were put on moistened filter paper in Petri dishes under ambient conditions. The structure of the seeds is shown in Figure 2. Seed weights were determined at the time of initiation of imbibition (0 h) and at 2-h intervals in the first 12 h and then 12-h intervals in the next 60 h. Ahead of each weighing, the seeds of different treatments were removed from the Petri dishes, blotted dry with new filter papers, weighed on a balance (to the nearest 0.001 g), and then returned to the moist filter paper. A completely randomized design was adopted in this trial. The increase in the mass of DS, BS, CS, EXS, and ENS was compared using imbibition curves.

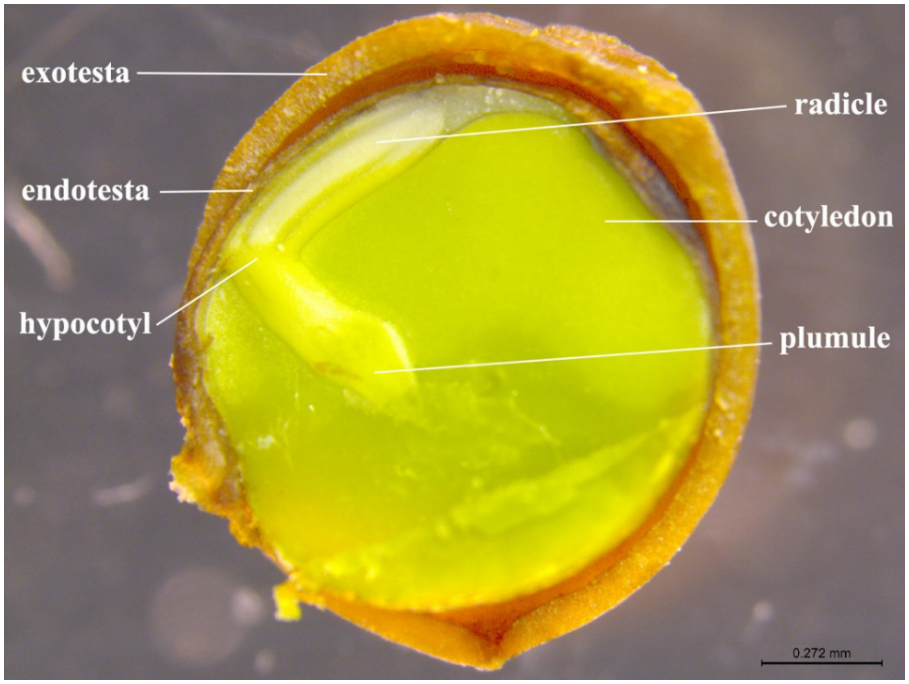


Figure 2. Longitudinal section along the raphe of Chinese pistache seeds.

2.2.3. Water Entry Site Identification

A water-blocking trial with a completely randomized design was carried out to detect the water entry sites. A total of 750 seeds of similar size were randomly divided into 5 groups with 150 seeds each. The following treatments were included in this trial: (1) whole seed exposed (AE); (2) whole seed exposed except for the hilum, raphe, and chalaza, which were sealed with nail glue (OE); (3) hilum exposed while all other areas are sealed with nail glue (HE); (4) raphe exposed while other areas are sealed with nail glue (RE); and (5) chalaza exposed while other areas are sealed with nail glue (CE). All treatments were repeated 3 times, with 50 seeds each replication. Prior to the above trial, intact Chinese pistache seeds were sealed with nail glue and placed on moist filter paper in a Petri dish, which showed that there was no seed swelling within 72 h, indicating that nail glue sealing can completely block seed swelling. The seeds of all treatments were placed in a Petri dish with moist filter paper, regularly observed and watered if necessary, and weighed every 4 h until the seed mass did not change.

2.3. Seed Respiration Rate

Four treatments of intact dry (DS), imbibed (BS), cracked (CS), and exotesta-removed (EXS) seeds were included in this trial, with a total of 600 seeds, three repetitions, and 50 seeds each. A completely randomized design was adopted in this test. A Li-6800 Portable Photosynthesis System (LI-COR Environmental, Lincoln, NE, USA) was utilized to measure the respiration rate of seeds with the supporting insect respiratory leaf chamber. During the measurements, the parameters were set as follows: flow rate of 1000 μmol s^{-1}, RH of 60%, temperature of 25 °C, and carbon dioxide concentration of 400 ppm.

2.4. Bioassay of Germination Inhibitors in Chinese Pistache Seeds

Three parts of the exotesta, endotesta, and embryo were obtained from dormant Chinese pistache seeds using a blade, which were quickly frozen with liquid nitrogen and stored in a freezer at 80 °C after grinding. A completely randomized design was adopted in this test.

In one experiment, the exotesta, endotesta, and embryo of the seeds were laid evenly underneath the moistened filter papers in the Petri dishes where the 60 cabbage seeds were sown per dish, with three repetitions. Nothing on the bottom of the Petri dishes was regarded as the control. The germination percentage of the cabbage seeds was calculated after 48 h of incubation.

In another experiment, the exotesta, endotesta, and embryo were added into 50-mL centrifugal tubes containing 50 mL of 80% methanol, which were placed in a closed refrigerator at 4 °C to extract the germination inhibitors from Chinese pistache seeds. During the extraction process, the centrifuge tube must be regularly shaken by ultrasonication to increase the extraction. After 24 h of extraction, the supernatant was filtered into a closed Erlenmeyer flask, and then, 80% methanol was added into the centrifuge tube to continue extracting, with three repetitions. Next, the methanol in the obtained supernatant was removed using a rotary evaporator at 40 °C. Extracted substances were washed with distilled water to a volume of 20 mL, with the same volume of distilled water as the control. Then, the germination inhibitors bioassay was carried out. Four samples of exotesta extracts, endotesta extracts, embryo extracts, and the control (distilled water) were acquired from the extraction process. Sixty cabbage seeds were sown on a disposable 120-mm Petri dish with filter papers moistened with 5-mL extraction solution or distilled water, with three repetitions. The test was carried out on an ultraclean workbench, and the Petri dishes were sealed with sealing film and placed in the tissue culture room. The number of germinating seeds was counted during the incubation process, and the seedling height and root length of the cabbage were measured after 72 h.

2.5. Measurements of Potential Inhibitors in Exotesta-Removed Seeds during Germination by Widely Targeted Metabolomics

Intact dry (DS′) and germinated (GS′) exotesta-removed seeds with three biological replications were included in this test. Dissolve 50 mg of lyophilized powder with 1.2-mL 70% methanol solution and vortex 30 s every 30 min for 6 times in total. Following centrifugation at 12,000 rpm for 3 min, the extracts were filtrated (SCAA-104, 0.22-μm pore size; ANPEL, Shanghai, China, http://www.anpel.com.cn/ [28]) before the UPLC-MS/MS analysis. The sample extracts were analyzed using an UPLC-ESI-MS/MS system (UPLC, SHIMADZU Nexera X2, https://www.shimadzu.com.cn/ [29]; MS, Applied Biosystems 4500 Q TRAP, https://www.thermofisher.cn/cn/zh/home/brands/applied-biosystems.html [30]). The analytical conditions were as follows: UPLC: column, Agilent SB-C18 (1.8 μm, 2.1 mm × 100 mm). The mobile phase consisted of solvent A, pure water with 0.1% formic acid, and solvent B, acetonitrile with 0.1% formic acid. Sample measurements were performed with a gradient program that employed the starting conditions of 95% A, 5% B. Within 9 min, a linear gradient to 5% A, 95% B was programmed, and a composition of 5% A, 95% B was kept for 1 min. Subsequently, a composition of 95% A, 5.0% B was adjusted within 1.1 min and kept for 2.9 min. The flow velocity was set as 0.35 mL per

minute; The column oven was set to 40 °C. The injection volume was 4 μL. The effluent was alternatively connected to an ESI-triple quadrupole linear ion trap (QTRAP)-MS.

The ESI source operation parameters were as follows: source temperature 550°C; ion spray voltage (IS) 5500 V (positive ion mode)/−4500 V (negative ion mode); ion source gas I (GSI), gas II (GSII), and curtain gas (CUR) set at 50, 60, and 25 psi, respectively; and the collision-activated dissociation (CAD) was high. Instrument tuning and mass calibration were performed with 10 and 100 μmol/L polypropylene glycol solutions in the QQQ and LIT modes, respectively. QQQ scans were acquired as MRM experiments with the collision gas (nitrogen) set to medium. DP (declustering potential) and CE (collision energy) for individual MRM transitions were done with further DP and CE optimization. A specific set of MRM transitions were monitored for each period according to the metabolites eluted within this period.

2.6. Investigating the Effects of Different Treatments on the Germination

To explore which factor hindered the germination of Chinese pistache seeds, the germinating test was conducted with BS, EXS, SAS (98% concentrated sulfuric acid-soaked seeds with a soaking time of 7 h), REC (exotesta and 1/2 cotyledon-removed seeds obtained by cutting longitudinally along the raphe and retaining 1/2 endotesta), GA (100 mg/L GA_3-treated seeds with a soaking time of 24 h), and CDS (cold-stratified seeds stratified with wet sand at 4 °C for approximately 100 days). A completely randomized design was adopted in this study. Sowing was conducted on moistened filter paper in Petri dishes under incubator conditions, with a photoperiod of 16-h light and 8-h dark at 25 ± 1 °C, relative humidity of 65%, and light intensity of 340 mol m^{-2} s^{-1}. All treatments were repeated for three times, with 30 seeds each replication. The soaking time in concentrated sulfuric acid and the concentration of GA_3 were based on previous results (Guo, unpublished data). The seeds were regularly watered, and the germination circumstances were regularly observed. After 30 days, the germination rate of the seeds in each treatment was calculated.

2.7. Morphological Observation during Seed Germination Process

The morphological characteristics of the seeds during germination were explored in this experiment. In the process of seed germination under different treatments, DS, BS, CS, and GS (germinated seeds) were sampled, with 20 seeds each. Among them, 10 seeds were observed and photographed by a stereomicroscope in a timely manner, while the remaining 10 seeds were immediately stored in a 80 °C freezer. Then, the stored seeds were sufficiently dried by a vacuum freeze dryer, bonded to the sample holder, sputtered with gold, and observed and photographed by a HITACHI S-3400N scanning electron microscope (HITACHI, Tokyo, Japan).

2.8. Statistical Analysis

A completely randomized design was adopted in this study. All experiments were carried out in triplicate, and the mean values with standard errors were used in this study. The data were analyzed by one-way ANOVA using IBM SPSS Statistics 23.0 (SPSS, Chicago, IL, USA). Duncan tests were applied to determine the differences between the treatments, and all tests were performed at the $\alpha = 0.05$ level.

3. Results

3.1. Morphological Variations of Chinese Pistache Seeds during Germination Process

During the germination process, the morphology of the Chinese pistache seeds changed greatly. The intact dry seeds (DS) were dehydrated, with a smaller volume and thicker exotesta of 0.082 mm, and the embryo was small and undifferentiated (Figure 3a,e,i,m,q). The size of imbibed seeds (BS) was slightly larger than that of DS; the raphe was cracked after imbibition for 72 h, and the embryo was swelled; and there was nearly no change in the exotesta thickness (0.080 mm; Figure 3b,f,j,n,q). Based on Figure 3c,g,k,o,q, the volume of cracked seeds (CS) increased evidently; the crack at the raphe expanded to 0.20 mm, and

the plumule was developed and differentiated to a certain extent; and the exotesta thickness decreased significantly to 0.07 mm. For germinated seeds (GS), the volume reached the maximum, and the radicle broke through the seed coat and extended; the plumule was differentiated to a large extent and had a prototypical leaf; and the exotesta thickness continued to decrease to a mean value of 0.064 mm (Figure 3d,h,l,p,q).

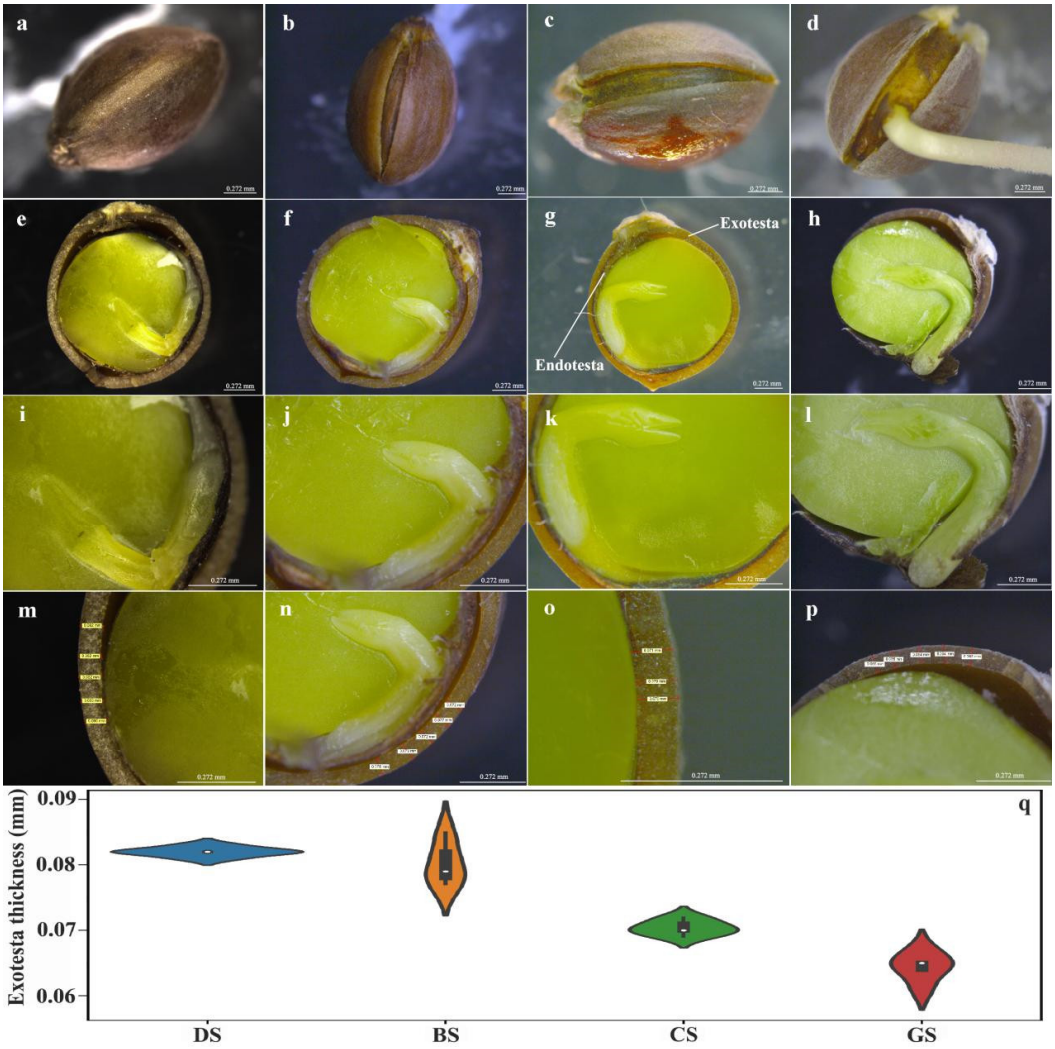


Figure 3. Morphology of Chinese pistache seeds during germination under a stereomicroscope. Raphe of intact dry (DS), imbibed (BS), cracked (CS), and germinated (GS) seeds (**a**–**d**); longitudinal section of DS, BS, CS, and GS (**e**–**h**); embryos of DS, BS, CS, and GS (**i**–**l**); exotesta section of DS, BS, CS, and GS (**m**–**p**); and exotesta thickness of DS, BS, CS, and GS (**q**).

The scanning electron microscope captured more detailed changes during the germination process (Figures 4 and 5). The longitudinal section and plumule of DS, BS, CS, and GS showed that the embryo was imbibed and differentiated gradually (Figure 4a–h). The inner cotyledons of DS and BS were smoother, with some linear bumps (Figure 4i,j), whereas there were some hollows and dark spots on the surfaces of the inner cotyledons of CS and multiple cracks on those of GS (Figure 4k,l). For the outer cotyledon, the surfaces of DS and BS were flatter, with regular cell shapes; however, there were some dark hollows between the cell walls, and many black spots appeared in the whole cells of CS, while more hollows and larger black spots were observed on the surface of the outer cotyledon of GS with an irregular cell shape (Figure 4m–p). The exotesta surface of DS seems compact; several small holes appeared on the BS surface; and more holes were found for CS and GS. Moreover, there was irregular block damage on the seed coat surface of GS (Figure 5a–d).

During the germination process, the hilum was looser and gradually became polyporous to provide more water and oxygen to seeds (Figure 5e–h). According to Figure 5i–l, with the progression of the germination, the exotesta became thinner, and more openings appeared.

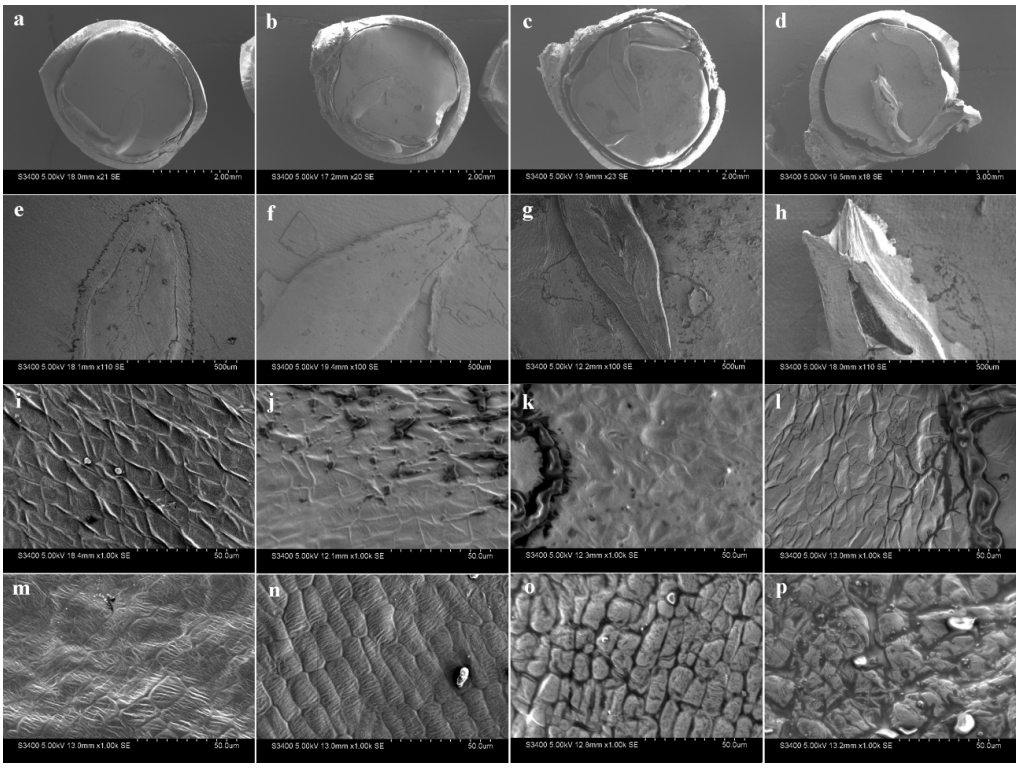


Figure 4. Longitudinal section of intact dry (DS), imbibed (BS), cracked (CS), and germinated (GS) seeds of Chinese pistache (**a**–**d**); plumule of DS, BS, CS, and GS (**e**–**h**); inner cotyledon of DS, BS, CS, and GS (**i**–**l**); and outer cotyledon of DS, BS, CS, and GS (**m**–**p**) under a scanning electron microscope.

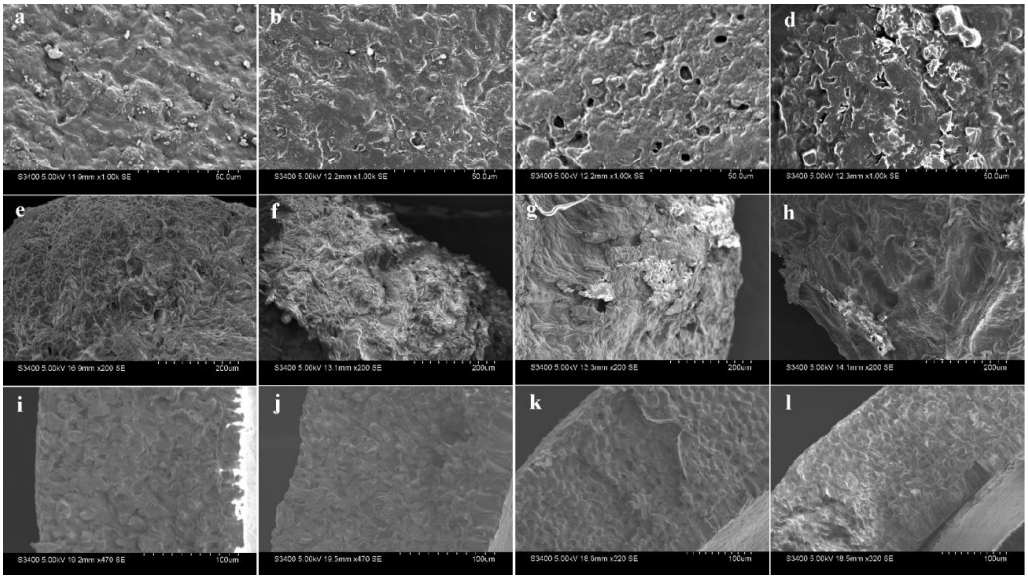


Figure 5. Exotesta of intact dry (DS), imbibed (BS), cracked (CS), and germinated (GS) seeds of Chinese pistache (**a**–**d**); hilum of DS, BS, CS, and GS (**e**–**h**); and exotesta section of DS, BS, CS, and GS (**i**–**l**) under a scanning electron microscope.

3.2. Water Permeability Evaluation of Chinese Pistache Seeds

The water content of intact Chinese pistache seeds first rapidly and then slowly increased with the prolonged absorption time and reached a plateau after 49 h. In the first 4 h, the seed water content increased rapidly by 12.32% (with an initial water content of 8.38%). From 4 h to 49 h, there was a slow increase in the water content of 10.29%. Afterward, the seed water content barely increased. When absorbing water for 72 h, the seeds seemed to reach saturation. During the whole absorption process, the total water absorption was 23.44% (Figure 6a).

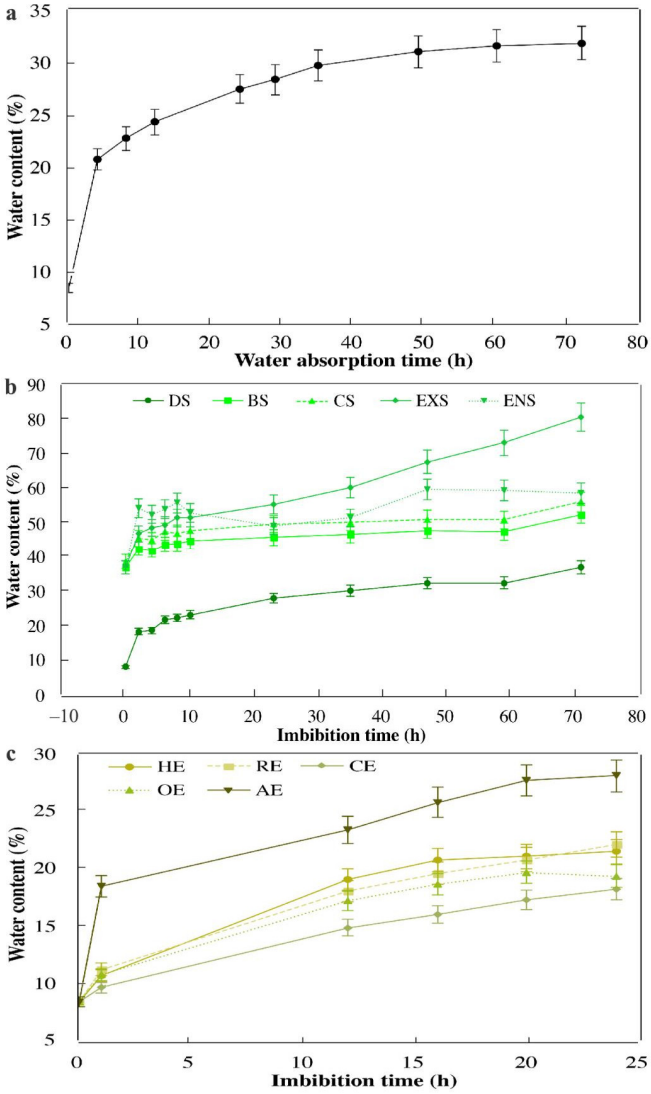


Figure 6. Variation in the water content of intact dried Chinese pistache seeds with the absorption time (**a**). Variation differences in the water content under different imbibition treatments (**b**). Variation differences in the water content under different blocking treatments (**c**). DS, intact dry seeds; BS, imbibed seeds; CS, cracked seeds; EXS, exotesta-removed seeds; ENS, endotesta-removed seeds; AE, whole area of the seed exposed; OE, whole area exposed except for the hilum, raphe, and chalaza; HE, hilum exposed; RE, raphe exposed; and CE, chalaza exposed.

In the water imbibition test, due to different seed coat weakening treatments or different stages in the germination process, the seeds under five treatments had various initial water contents ranked as CS (38.9%) > BS (37.2%) = EXS = ENS > DS (8.38%). The water content variation presented fast at first, and then, the trends increased slowly, except for ENS. The ENS treatment showed a fluctuating trend with the increasing imbibition

time. After 35 h of imbibition, the seed water content increased by 16.32% under the EXS treatment, which was higher than that under the other treatments. When imbibing for 71 h, the seed water content ranked as follows: EXS (80.76%) > ENS (58.77%) > CS (56.33%) > BS (52.71%) > DS (37.20%) (Figure 6b).

The water entry sites of Chinese pistache seeds were identified in this test. The water content of the seeds showed a similar tendency of first increasing rapidly and then slowly increasing under all treatments. The initial moisture content of the seeds was 8.38%. After 2 h of imbibition, the water content of the AE treatment increased rapidly by 10.16% and was higher than that of the other treatments throughout the imbibition process. The water content of the seeds in the HE and RE treatments was similar, and their total water absorption content was 13.16% and 13.77%, respectively. The moisture content of CE was lower than that of the other treatments during the whole imbibition process. The total water absorption of each treatment ranked as follows: AE (19.82%) > RE (13.77%) > HE (13.16%) > OE (11.02%) > CE (9.90%) (Figure 6c).

3.3. Effects of Different Treatments on the Respiration Rate of Chinese Pistache Seeds

The change in the respiration rate of Chinese pistache seeds under different treatments was shown in Figure 7a. CS had the highest respiration rate of 440.14 μg CO_2 g^{-1} min^{-1} among all treatments, followed by EXS (281.71 μg CO_2 g^{-1} min^{-1}), which were 94.86 and 60.71 times that of DS and 21.39 and 13.69 times that of BS, respectively. DS had the lowest respiration rate of 4.64 μg CO_2 g^{-1} min^{-1} (Figure 7a).

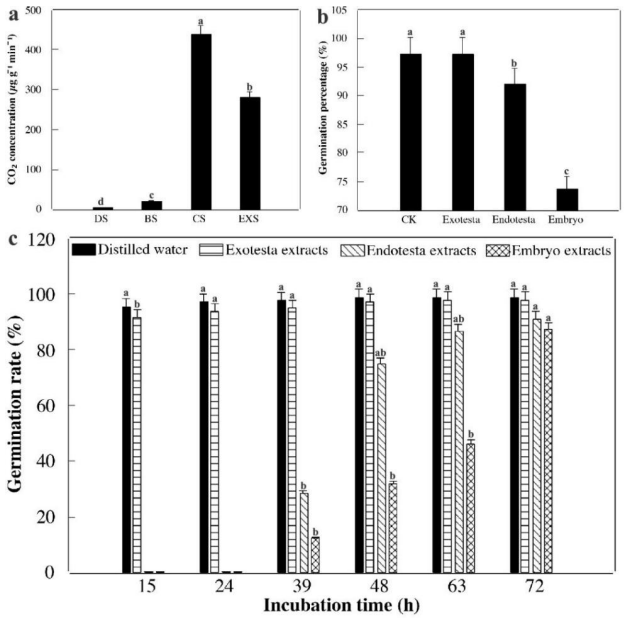


Figure 7. Variation in the respiration rate of Chinese pistache seeds under different treatments (**a**). Germination percentage of cabbage seeds under different treatments (**b**). CK, Exotesta, Endotesta, and Embryo mean nothing, exotesta, endotesta, and the embryos of Chinese pistache seeds laid on the bottom of the Petri dishes, respectively. Variations in the germination rate of cabbage seeds under different treatments with the increasing incubation time (**c**). Distilled water, exotesta extracts, endotesta extracts, and embryo extracts of Chinese pistache seeds were added to Petri dishes with filter papers to cultivate cabbage seeds. Different letters over bars indicate significant differences.

3.4. Inhibition by Chinese Pistache Seed Extracts of Cabbage Seed Germination, Seedling Height and Root Length

The germination percentage of cabbage seeds of different treatments is shown in Figure 7b. The exotesta and control (CK) treatments had the highest germination percentage of 97.37%, followed by the endotesta treatment (92.11%, $p < 0.05$), whereas the embryo treatment had the lowest germination percentage (73.68%) with a significant difference

from the other three treatments ($p < 0.05$). The endotesta and embryos of Chinese pistache seeds underneath the moistened filter papers on the bottom of the Petri dishes significantly reduced the germination percentage of cabbage seeds by 7.89% and 26.32%, respectively.

The germination rate of cabbage seeds incubated with distilled water, exotesta extracts, endotesta extracts, and embryo extracts of Chinese pistache seeds presented different variation tendencies with a prolonged incubation time (Figure 7c). After 24 h of incubation, almost all cabbage seeds with distilled water and exotesta extracts completed the germination process, with no striking difference. However, endotesta and embryo extract-treated cabbage seeds initially germinated after incubating for 39 h, and the germination rate with embryo extracts was significantly lower than the others ($p < 0.05$), although there were no significant differences among the four treatments after incubating for 72 h. The final germination percentage was the highest in distilled water-treated cabbage seeds (98.89%), which was 11.67% higher than cabbage seeds treated with embryo extracts.

The seedling height and root length of cabbage seeds incubated with distilled water, exotesta extracts, endotesta extracts, and embryo extracts of Chinese pistache seeds are shown in Figure 8a. Cabbage seeds treated with distilled water had the highest seedling height of 0.59 cm and differed significantly from the other treatments ($p < 0.05$). Embryo extract-treated cabbage seeds showed the lowest seedling height of 0.20 cm, with no difference from the endotesta extracts. Similar to the seedling height variation, the maximum root length (1.60 cm) was observed in cabbage seeds incubated with distilled water, which was 2.40 times that of the embryo extract treatment (0.67 cm). Moreover, the growth characteristics of the distilled water and exotesta extracts treatments were very similar, while the other two treatments, especially the embryo extracts, had worse growth (Figure 8b).

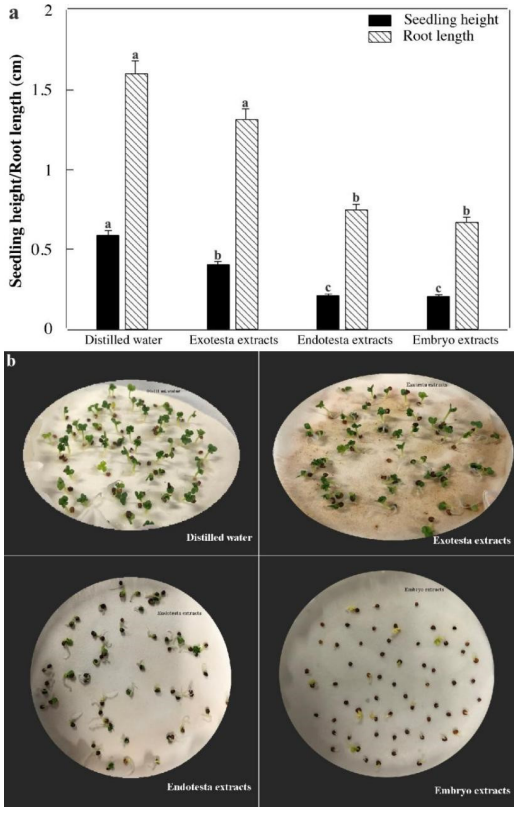


Figure 8. Seedling height and root length of cabbage seeds under different treatments. Different letters over bars indicate significant differences (**a**). Distilled water, exotesta extracts, endotesta extracts, and embryo extracts of Chinese pistache seeds were added to Petri dishes with filter papers to cultivate cabbage seeds. Growth characteristics of cabbage under different treatments after incubation for 72 h (**b**).

3.5. Identification of Endogenous Inhibitors of Chinese Pistache Seeds

The widely targeted metabolomics was adopted to explore the potential endogenous inhibitors of Chinese pistache seeds during germination. PCA indicated a distinct separation between the two samples (DS′ and GS′) and strong uniformity among the three biological replicates (Figure 9a). The results indicated that the metabolome data had good repeatability and reliability. In total, 856 metabolites were identified; among which, 294 were upregulated and 92 were downregulated significantly (Figure 9b). The potential endogenous inhibitors may be included in the downregulated metabolites. Based on the KEGG analysis, these metabolites were significantly enriched in the metabolic pathways, biosynthesis of the secondary metabolites, purine metabolism, etc. (Figure 9c). In addition, 30 potential inhibitors were screened according to the fold change value between DS′ and GS′, such as 2-oxoadipic acid, ferulic acid, ABA, etc. (Table 1).

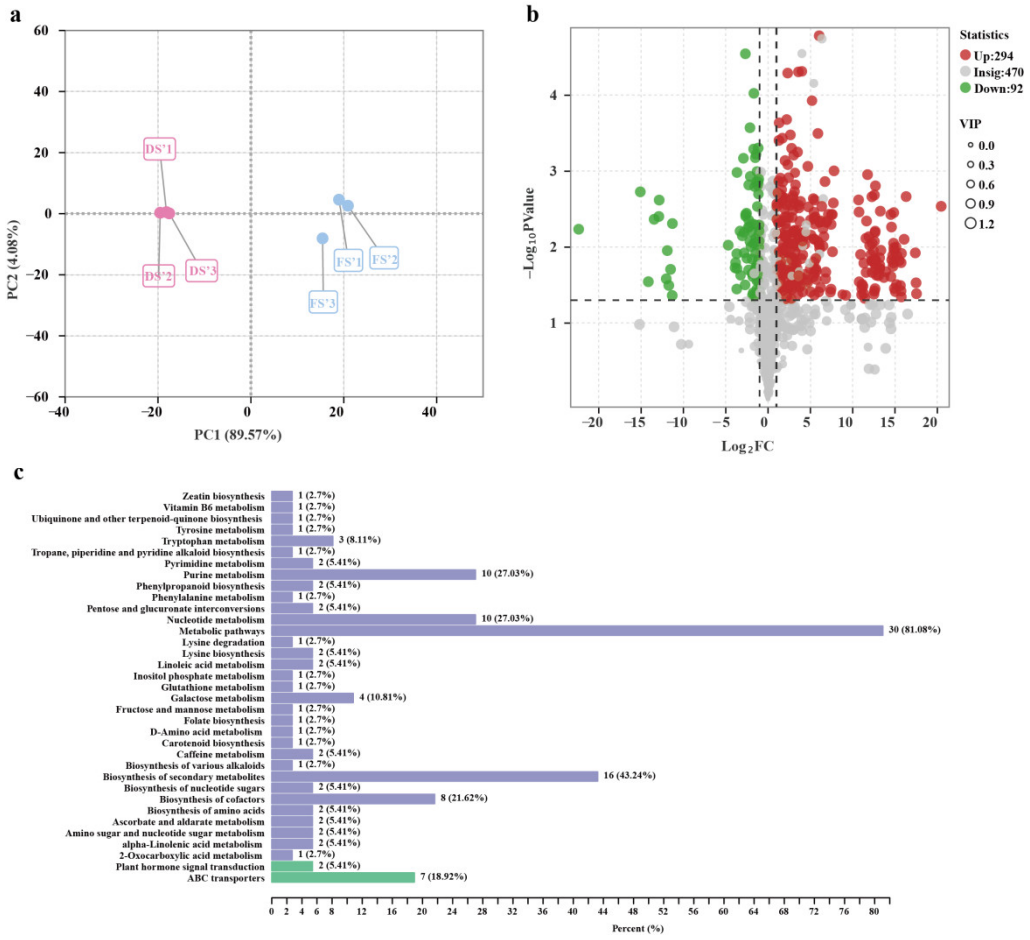


Figure 9. Principal component analysis (PCA) clustering based on metabolome data (**a**). Differential metabolite volcanic map (**b**). KEGG classification map of downregulated metabolites (**c**). DS′, exotesta-removed dry or dormant seeds; GS′, exotesta-removed germinated seeds.

3.6. Effects of Different Treatments on the Germination of Chinese Pistache Seeds

After 30 days of incubation, the seeds with the exotesta and 1/2 cotyledon removed (REC) had the highest germination rate (89%), followed by those soaked in concentrated sulfuric acid (SAS, 50%), GA_3-treated seeds (GA, 45%), exotesta-removed seeds (EXS, 40%), and cold-stratified seeds (CDS, 38.6%), while none of the imbibed seeds (BS) germinated. REC significantly improved the germination, and the germination rate was up to 89% after 7 days of incubation. The seeds of the SAS treatment began to germinate on the 7th day after incubation, and those of GA, EXS, and CDS treatment began on the 10th day. The germination rate of the SAS and GA treatments increased rapidly from 10 days to 15 days

of incubation, while EXS and CDS increased rapidly from 15 days to 20 days of incubation. The germination rate of REC was obviously higher than those of the other treatments. There was no visible difference between EXS and CDS (Figure 10).

Table 1. Potential endogenous inhibitors of Chinese pistache seeds by fold change.

Compounds	Formula	Class I	Relative Content		Fold_Change
			DS′	GS′	
2-Hydroxy-3-phenylpropanoic acid	$C_9H_{10}O_3$	Phenolic acids	1.64×10^5	9.00	5.50×10^{-5}
Myricetin-3-O-β-D-glucoside	$C_{21}H_{20}O_{13}$	Flavonoids	3.46×10^4	9.00	2.60×10^{-4}
2-Oxoadipic acid	$C_6H_8O_5$	Organic acids	2.28×10^4	9.00	3.95×10^{-4}
2,5-Dihydroxybenzoic acid; Gentisic Acid	$C_7H_6O_4$	Phenolic acids	2.04×10^6	1.37×10^5	6.69×10^{-2}
3,4-Dihydroxybenzoic acid (Protocatechuic acid)	$C_7H_6O_4$	Phenolic acids	3.15×10^6	2.20×10^5	7.01×10^{-2}
3-(4-Hydroxyphenyl)-propionic acid	$C_9H_{10}O_3$	Phenolic acids	2.75×10^5	2.11×10^4	7.69×10^{-2}
2,3-Dihydroxybenzoic Acid	$C_7H_6O_4$	Phenolic acids	3.65×10^6	2.82×10^5	7.73×10^{-2}
Ferulic acid	$C_{10}H_{10}O_4$	Phenolic acids	1.87×10^5	1.46×10^4	7.84×10^{-2}
Abscisic acid	$C_{15}H_{20}O_4$	Organic acids	1.73×10^5	1.36×10^4	7.87×10^{-2}
DL-3-Phenyllactic acid	$C_9H_{10}O_3$	Organic acids	2.09×10^5	2.19×10^4	1.05×10^{-1}
4,8-Dihydroxyquinoline-2-carboxylic acid	$C_{10}H_7NO_4$	Organic acids	2.64×10^5	7.05×10^4	2.67×10^{-1}
Apigenin-8-C-(2″-glucosyl)arabinoside	$C_{26}H_{28}O_{14}$	Flavonoids	5.71×10^4	1.56×10^4	2.73×10^{-1}
Syringaldehyde; 4-Hydroxy-3,5-Dimethoxybenzaldehyde	$C_9H_{10}O_4$	Phenolic acids	8.05×10^4	2.36×10^4	2.94×10^{-1}
2-(Formylamino)benzoic acid	$C_8H_7NO_3$	Phenolic acids	1.72×10^5	5.26×10^4	3.06×10^{-1}
Ursonic acid	$C_{30}H_{46}O_3$	Terpenoids	1.36×10^6	4.16×10^5	3.07×10^{-1}
Vanillin; 4-Hydroxy-3-Methoxybenzaldehyde	$C_8H_8O_3$	Phenolic acids	3.96×10^5	1.29×10^5	3.25×10^{-1}
2,6-Diaminooimelic acid	$C_7H_{14}N_2O_4$	Organic acids	1.90×10^5	6.26×10^4	3.31×10^{-1}
N1,N8-Bis(sinapoyl)spermidine	$C_{29}H_{39}N_3O_8$	Alkaloids	1.84×10^5	6.51×10^4	3.54×10^{-1}
Pyridoxine	$C_8H_{11}NO_3$	Others	1.58×10^5	5.73×10^4	3.63×10^{-1}
Caffeic aldehyde	$C_9H_8O_3$	Phenolic acids	8.63×10^4	3.48×10^4	4.03×10^{-1}
N-[7′-(4′-Methoxyphenyl)ethyl]-2-methoxybenzamide	$C_{17}H_{19}NO_3$	Alkaloids	4.91×10^4	2.06×10^4	4.19×10^{-1}
3-(8,11-Heptadecadienyl)-1,2-benzenediol	$C_{23}H_{36}O_2$	Phenolic acids	8.13×10^4	3.46×10^4	4.25×10^{-1}
Ambolic acid	$C_{31}H_{50}O_3$	Terpenoids	1.40×10^4	5.94×10^3	4.26×10^{-1}
4-Hydroxybenzoic acid	$C_7H_6O_3$	Phenolic acids	4.79×10^5	2.17×10^5	4.53×10^{-1}
Ursolic acid	$C_{30}H_{48}O_3$	Terpenoids	2.25×10^6	1.02×10^6	4.54×10^{-1}
4,7,9,9′-Tetrahydroxy-3,3′-dimethoxy-8-O-4′-neolignan	$C_{20}H_{26}O_7$	Phenolic acids	3.55×10^4	1.68×10^4	4.73×10^{-1}
Secoisolariciresinol 4-O-glucoside	$C_{26}H_{36}O_{11}$	Lignans and Coumarins	1.87×10^5	8.93×10^4	4.79×10^{-1}
Moronic acid	$C_{30}H_{46}O_3$	Terpenoids	8.05×10^5	3.92×10^5	4.87×10^{-1}
Jasmonic acid	$C_{12}H_{18}O_3$	Organic acids	5.83×10^4	2.90×10^4	4.96×10^{-1}

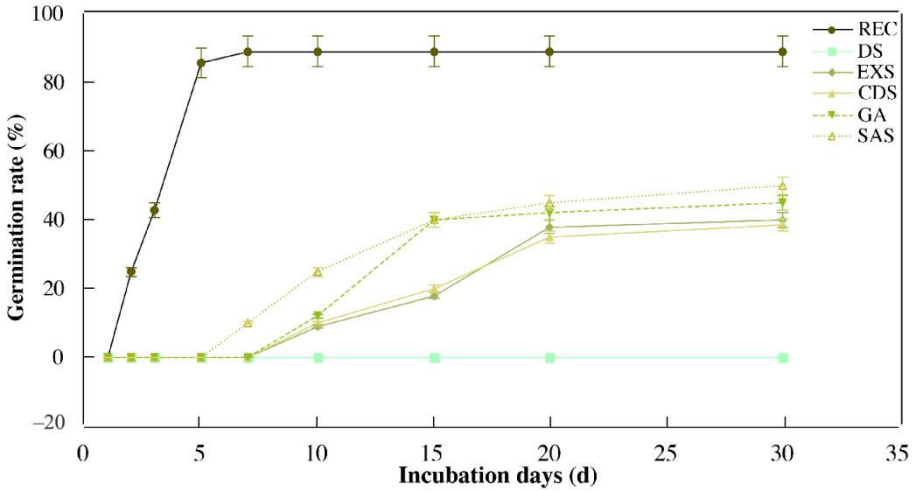


Figure 10. Variations in the germination rate of Chinese pistache seeds under different treatments. BS, imbibed seeds; EXS, exotesta-removed seeds; SAS, concentrated sulfuric acid-soaked seeds; REC, seeds with the exotesta and 1/2 cotyledon removed; GA, seeds treated with 100 mg/L GA_3; and CDS, seeds treated with stratified wet sand at 4 C for approximately 100 days.

4. Discussion

This study clarified the reasons or characteristics for the dormancy of Chinese pistache seeds from an integrated perspective, covering the water permeability, respiration, and germination inhibitors. It showed the morphological variations of different tissues of Chinese pistache seeds during the germination process. The water permeability of different tissues and sites and respiration rate were evaluated. Germination inhibitors were identified by bioassay and widely targeted metabolomics. These results indicated that the permeability and inhibitors could affect seed germination. Additionally, the effects of different treatments on the germination were explored to further verify our results.

The testa of Chinese pistache seeds was water-permeable, and the removal of testa improves the imbibition rate (Figure 6a,b). The structure and substances in the seed coat can protect the seeds from stressful environmental conditions upon germination. For example, flavonoids in the testa could limit the imbibitional damage by decreasing the testa permeability, thus controlling the rate of water uptake [16]. Seed scarification could accelerate the water entry into seeds, thus promoting metabolic reactions in the embryo and the cotyledons [21]. The scarification of *Pistacia atlantica* Desf. seeds kickstarted germination after 20 days of cultivation and had a positive effect on the germination rate [22]. Li and Min (2020) also found that the testa of *Phoebe bournei* (Hemsl.) Yang seeds was water-permeable, suggesting that they are not part of the physical dormancy; however, the seed coat of *Tilia miqueliana* Maxim exhibits poor water permeability due to its complex multilayer and hard and dense cell structure [31,32]. Therefore, ultrastructural research also plays a vital role in the water permeability of seeds [33,34]. Sclereids lead to poor water uptake and provide mechanical constraint due to their thickened secondary cell walls [35]. The properties of hard seed envelopes rely strongly on the composition and arrangement of sclerenchyma [36]. Water permeability among different sites of seeds may be greatly different. In this study, the raphe and hilum areas contributed most to water imbibition (Figure 6c). This may be closely related to the physicochemical characteristics of the testa of Chinese pistache seeds, including the looser and polyporous hilum compared to other sites (Figure 5e). In addition, the light color of the raphe may be related to its permeability [37]. Jaganathan et al. (2019) found that imbibition primarily occurs at the hilum and extrahilar region of *Astragalus adsurgens* Pall seeds [38]. Finch-Savage and Leubner-Metzger (2006) found that oilseeds contain less bound water when equilibrated at any given relative humidity. The cotyledons of Chinese pistache seeds have more than 50% oil, which may affect the water uptake and germination [39].

In the seed respiration test, there were significant differences among the four treatments. CS had the highest respiration rate, followed by EXS, BS, and DS (Figure 7a). CS was possibly in a plateau phase (phase II) of water uptake. This phase encompasses mitochondrial and DNA repair, de novo mRNA synthesis and translation, enhanced respiration/metabolism, etc. [33]. An increase in mitochondrial respiration may support ATP synthesis [40]. These events in seeds may have led to an increased respiration in CS. Additionally, removal or scarification of the exotesta may weaken the covering layer, increase the water uptake, and improve the oxygen availability to the embryo. The removal of exotesta can also promote the release of endogenous germination inhibitors, further enhancing seed respiration [40]. This could explain why EXS has a higher respiration rate in this study. The sensitivity of spinach (*Spinacia oleracea* L.) seed germination to moisture is driven by the oxygen availability to the seed embryo [41]. The inhibitory effects of phenolic compounds on seed germination are closely related to the epicarp permeability and oxygen supply to the embryo [42,43]. Clipping the seeds or completely removing the seed coat apparently relieved any mechanical restraint and/or barriers to the gas exchange, as these treatments greatly improved the germination of 'Genesis' triploid watermelon (*Citrullus lanatus* (Thunb.) Matsum & Nakai) compared to intact seeds [42]. In addition, Borisjuk and Rolletschek (2009) found that the major determinant of a diffusive oxygen uptake was the chemical composition of the layer covering the oil-storing embryo rather than the tissue thickness [43]. However, respiratory inhibitors promoted the germination of

Zinnia elegans Jacq seeds, which surely inhibited the O_2 uptake of the seeds. This suggested that such promotional effects are not mainly attributable to the increased O_2 level and that oxidative respiration is not a rate-limiting step for seed germination at least 48 h after imbibition [40]. Indeed, during seed development and germination, there is a mechanism of hypoxia perception and balance that can control the production and utilization of energy. The seeds of rice and barnyard grass can germinate and grow underwater. Under anoxic conditions, ATP begins to synthesize soon after imbibition, possibly through glycolysis, and finally, pyruvate is converted into ethanol [12].

Our results showed that the endotesta and embryo methanol extracts of Chinese pistache seeds, especially embryo extracts, could delay and inhibit the germination of cabbage seeds to some extent; they also significantly inhibited the seedling growth of cabbage (Figures 7b,c and 8). Therefore, the endotesta and embryos of Chinese pistache seeds may contain inhibitors that can be dissolved in methanol. Then, the exotesta-removed seeds were used to identify the germination inhibitors by the widely targeted metabolomics. The results revealed that phenolic acids, organic acids, alkaloids, terpenoids, and flavonoids may be the potential inhibitors of Chinese pistache seeds. Phenolic acids and organic acids share a large proportion (Table 1). Phenolic acids and flavonoids are the key secondary metabolites widely found in plants and exert antioxidant activities, and they contribute to the change in antioxidant activity during germination [44–46]. Phenolic compounds that act as antioxidants suppress seed germination in a dose-dependent manner. They may block activation of the pentose phosphate pathway. H_2O_2 oxidatively denatures germination inhibitors, such as ferulic and coumaric acids [40]. Ferulic acid as a possible germination inhibitor was also identified in this study (Table 1). The dormancy of Atlas pistachios (*Pistacia atlantica* Desf.) is localized at the level of the pericarp and kernel. This is explained by their high content of phenolic compounds, which limits the metabolic reactivation during germination [23]. Higher phenolic contents were observed in the epicarp than in the seeds of *Pistacia atlantica* Desf. subsp. *atlantica*, regardless of the ecotype [47]. Organic acids could also significantly inhibit *Polygonatum cyrtonema* Hua seed germination. Benzoic acid, ferulic acid, and coumaric acid might have a major inhibitory effect on the germination of *Polygonatum cyrtonema* seeds [46], which is similar to our results. Organic acids and flavonoids may participate in acidifying the endosperm tissue, releasing stored starch into the metabolism to affect seed germination [48]. Zhao et al. (2018) indicated that butylated hydroxytoluene (BHT) primarily led to dormancy in *Sapium sebiferum* (L.) Roxb. seeds [49].

Seed scarification, including REC, EXS, and SAS, greatly increased the germination rate of Chinese pistache seeds (Figure 10). This verified our results that the testa of Chinese pistache seeds negatively affected their germination by reducing the water permeability, decreasing the oxygen availability, and enhancing the mechanical barrier. The seeds of *Pistacia* species are surrounded by a hard sclerotic endocarp/exotesta that makes germination difficult, and scarification and cold stratification were found to improve seed germination in *Pistacia* spp. The highest germination rate was obtained by sulfuric acid scarification in *Pistacia khinjuk* Stocks. [21]. Clipping the seeds or completely removing the seed coat also greatly improved the germination of 'Genesis' triploid watermelon (*Citrullus lanatus*) by relieving any mechanical restraint and/or barriers to the gas exchange [42]. Seed germination was promoted by removal of the pericarp from the seeds or by the removal of ethanol-soluble compounds from the seeds with pericarp [40]. However, seed scarification (removal of the epicarp) did not modify the germination rate of Atlas pistachio (*Pistacia atlantica* Desf.) seeds regardless of the ecotype. In fact, the total phenolic compounds were present not only in the epicarp but also in the kernels, which may explain the poor germination after scarification [47]. In this study, bioassay tests indicated that the endotesta and embryos of Chinese pistache seeds may contain inhibitors that can be dissolved in methanol. The widely targeted metabolomics analysis revealed that phenolic acids and organic acids are major endogenous inhibitors in the endotesta and embryos of Chinese pistache seeds (Table 1). Thus, the highest germination of REC in this study (Figure 10)

may be attributed to the inhomogeneous distribution (content) of germination inhibitors between the embryos and the testa.

The GA_3 treatment and cold stratification also significantly increased the germination rate of Chinese pistache seeds compared with DS (Figure 10). GA would improve the embryo growth potential and overcome the mechanical barrier of seed envelopes during germination [50]. ABA and GAs are the main phytohormones that antagonistically regulate seed dormancy. Dormancy release and germination are accompanied by enhanced ABA degradation and increased GA biosynthesis [50–52]. ABA was identified as potential germination inhibitors in this study (Table 1). ABA can inhibit seed germination by affecting the cell cycle, which is related to the activation of a residual G1 kinase that becomes inactivated in the absence of ABA [52,53]. Additionally, ABA represses the expression of the genes involved in photosynthesis and ten metabolic pathways to suppress the secondary metabolism of the embryos, resulting in the inhibition of embryo germination [54]. GA_3 increased the germination percentage in Chinese pistache seeds from some parent plants but was not as effective as stratification [26]. However, GA_3 application at 500 ppm negatively affected seed germination of the *P. khinjuk*-A genotype [21]. Cold stratification activated gluconeogenic processes, increased the H_2O_2-scavenging enzyme activity, and consequently decreased hydrogen peroxide and lipid peroxidation, which subsequently increased the seed germination capacity of pistachios (*Pistacia vera* L.) [9]. Increases in the stratification periods were found to be positively correlated with the germination percentages of *P. vera* and *P. khinjuk* seeds [21]. Germination of the GA_3 treatment and cold stratification was lower than that of REC in this study, which could be explained in two aspects. On the one hand, the endotesta and embryo of Chinese pistache seeds contain various germination inhibitors; on the other hand, the exotesta may prevent inhibitor leakage from the embryo.

5. Conclusions

In conclusion, the testa of Chinese pistache seeds was water-permeable, and the raphe and hilum areas contributed most to the imbibition. Removal of the testa increased the imbibition rate and improved the oxygen availability to the embryo. The endotesta and embryo of the Chinese pistache seeds contain germination inhibitors, such as phenolic acids and organic acids. Seed scarification, exogenous GA_3, and cold stratification greatly increased the germination rate of Chinese pistache seeds, but seed scarification (removal of the exotesta and 1/2 cotyledon) was the most effective strategy. There seems to exist both a mechanical dormancy due to the testa and a physiological one due to the embryos. However, the detailed mechanisms of germination inhibitors and the further functional verification need to be explored in future research.

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Abbreviations

GA_3, gibberellin; ABA, abscisic acid; TTC, triphenyl tetrazolium chloride; DS, intact dry seeds; BS, imbibed seeds; CS, cracked seeds; EXS, exotesta-removed seeds; ENS, endotesta-removed seeds; AE, whole seed exposed; OE, whole seed exposed except

for the hilum, raphe and chalaza; HE, hilum exposed; RE, raphe exposed; CE, chalaza exposed; SAS, 98% concentrated sulfuric acid-soaked seeds; REC, exotesta and 1/2 cotyledon-removed seeds; GA, 100 mg/L GA_3-treated seeds; CDS, cold-stratified seeds; GS, germinated seeds; DS′, exotesta-removed dry seeds; and GS′, exotesta-removed germinated seeds.

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