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Effects of Different Treatments on Physiological Characteristics of *Cephalotaxus sinensis* Seeds

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Abstract

Cephalotaxus sinensis seeds can't germinate even in the appropriate environment. However, numerous studies have showed that cold stratification and gibberellin acid (GA) can break the seed dormancy and promote seed germination effectively. To investigate the effect of cold stratification and different concentrations of exogenous GA_3 on dormancy breaking in seeds of *Cephalotaxus sinensis*, we monitored germination rates and changes in soluble sugar, starch, amylase, soluble protein, free amino acid during cold stratification. The results showed that seeds stratified for 5 months germinated to 12.7%, while those disposed with 200, 400, 600 mg/L of GA_3 germinated to 29.2%, 21.7%, and 18.4%, respectively. Free amino acid content was enhanced significantly, whereas soluble sugar content decreased during 45 days and then increased constantly. Additionally, the main reserves such as starch, protein decreased significantly during cold stratification, and cold stratification induced increases in the activities of α-amylase, $(\alpha + \beta)$-amylase. The preliminary results show that the combination of GA_3 and cold stratification has better effect to break seed dormancy.

Keywords

Cephalotaxus sinensis, Cold Stratification, Seed Germination, Reserves, Enzyme

1. Introduction

Seeds do not have the capacity of germination even in the presence of favorable environmental conditions [1]. It is a common phenomenon in seeds of many woody plant species and the degree of seed dormancy varies both among and within species [2]. The timing of germination is determined to a large extent by the depth of seed dormancy. If there is a wide range of environments under which seeds are capable of germination, the seed dormancy is considered to be shallow [3]. Seed dormancy is an innate seed

property that prevents seed germination in a specified period of time, under any combination of physical environmental factors [4]. It plays an important role in the survival of individual plants, species continuation and evolution. But it hinders the development of the forestry production to some extent.

Cold stratification and treatment with exogenous hormones have been used to break seed dormancy. Cold stratification at 5˚C is the optimal temperature for mostly species, but sometimes it will fluctuate with different species [5]. The deep dormancy of apple seeds is broken by cold stratification at a temperature of 5˚C for three months which makes the seeds germinate quickly and evenly [6]. Also, Pei *et al.* [7] pointed out that cold stratification had a significant effect to promote seeds germination and seedling growth in the following growing season. Additionally, researchers usually combined GA with cold stratification to promote germination rate [8] [9]. Cold stratification for breaking seed dormancy will lead to a series of complex interactions changes in cells. Some reports pointed out that biochemical and structural changes will appear in seeds in the process of cold stratification [10]. By studying effects of moist cold stratification on germination, plant growth regulators and metabolites, Chen *et al.* [11] indicated that cold stratification has an important influence on enzymatic activities and reserves in seeds.

As a Tertiary relict plant, *Cephalotaxus sinensis* is an endemic species to China [12]. Although it is naturally distributed in the Yangtze River and its south region, *Cephalotaxus sinensis* has a strong cold resistance and it can be exposed to winter in the Beijing area [13]. It has a wide range of uses. For example, it can be cultivated as an ornamental plant in the garden and it can be trained as a bonsai by trimming and modeling. Additionally, it contains a variety of bioactive substances, which makes it has broad application prospects in the fields of medicine, toxicology and phytochemistry [14]-[16]. Seeding propagation is generally used in production of *Cephalotaxus sinensis*, but the seeds remain deeply dormant in natural condition, and they will not germinate until the next or third year after landing [17] [18]. Therefore, it is important to study the methods of seed dormancy releasing.

The aim of the research is to test the changes of enzymatic activities, and reserves during cold stratification in seeds, and study the influence of cold stratification and exogenous hormones GA_3 on seed dormancy and germination.

2. Materials and Methods

2.1. Seed Sources

Seeds were obtained from Funiu Mountain of Henan province, China, on the hillside with the altitude of 700 meters. After being collected from adult trees in September 2014, the seeds were air-dried at room temperature and stored containers at 2˚C - 5˚C.

2.2. Cold Stratification

The experiments were conducted in test plots of Shandong Agricultural University. To determine the response of seeds to cold stratification, seeds were soaked for 24 h with

water, 200, 400, 600 mg/L of GA_3, respectively, and mixed with moistened sand in a ratio of 1:3. After that, the mixture of seeds and sand was buried in leeward side for the depth of 50 cm for 5 months. The moisture of seeds and sand were checked to avoid oxygen deprivation and water shortage of sand so that the seeds would not get mouldy during the entire period of stratification. Experiments were conducted every 15 days.

2.3. Analysis for Soluble Sugar and Starch

Soluble sugar was extracted from 1 g FM of seed in 25 ml of boiling water. After heating the seeds for 10 minutes at 100˚C, the extract was filtered through filter paper, diluted with water to 50 ml and mixed. Then 0.5 ml of extract, 1.5 ml of distilled water, 0.5 ml of 2% anthrone solution, and 5 ml of H_2SO_4 were mixed for 10 minutes. The soluble sugar was measured by a spectrophotometer at a wavelength of 620 nm, following the methods for extraction and preparation of test solutions and standards of Li [19]. There were three replications of each sample.

After heating the remaining seeds with 20 ml water for 15 minutes at 100˚C, the extract was mixed with 2 ml of 9.2 mol/L per chloric acid. Then the remaining steps are the same as soluble sugar.

2.4. Analysis for Amylase

Extraction procedures of Li [19] were adopted for the determination of amylase activity. For the preparation of enzyme extract, 1 g seeds were homogenized with 8 ml of water. After 20 minutes' standing, the extract was centrifuged at 10,000 r/min for 20 min, filtered through two layers of filter paper to remove impurities. All the preparations were carried out at 4˚C. The supernatant obtained was used as crude enzyme extract for α-amylase and $\alpha + \beta$-amylase assay. Then the detailed steps are shown in **Table 1**.

After heating the mixture for 5 min at 100˚C, the enzyme activity was measured by a spectrophotometer at a wavelength of 540 nm.

Table 1. The reagent content and operating sequence of enzyme activity assay.

Action item	Number					
	I-1	I-2	I-3	II-1	II-2	II-3
Amylase stock solution/ml	1.0	1.0	1.0	0	0	0
Passivate β-amylase	Heat the supernatant for 15 min at 70˚C					
Amylase stock solution/ml	0	0	0	1.0	1.0	1.0
3,5-dinitrosalicylic acid/ml	2.0	0	0	2.0	0	0
Pre heat preservation	Heat the mixture for 10 min at 40˚C					
1% starch solution/ml (40˚C)	1.0	1.0	1.0	1.0	1.0	1.0
Heat preservation	Heat the mixture for 5 min at 40˚C					
3,5-dinitrosalicylic acid/ml	0.0	2.0	2.0	0.0	2.0	2.0

2.5. Analysis for Protein

The soluble protein content was measured by using Coomassie brilliant blue staining referring to extraction procedures of Li [19]. 1 g seeds were ground with 8 ml of water and then the extract was centrifuged at 10,000 r/min for 20 min. 1ml of protein stock solution was mixed with 5 ml of Coomassie brilliant blue, measured by a spectrophotometer at a wavelength of 595 nm.

2.6. Analysis for Free Amino Acid

1 g seeds were ground with 5 ml of 10% acetic acid, diluted with water to 100 ml, and the extract was filtered through a layer of filter paper to remove impurities. Then 1 ml of filtrate, 1 ml of ammonia-free distilled water, 3 ml of ninhydrin, 0.1 ml of 0.1% ascorbic acid were mixed and heated for 15 min at 100˚C. After diluting with 60% ethanol to 20 ml, the mixture was measured by a spectrophotometer at a wavelength of 570 nm.

2.7. Analysis for Crude Fat

Crude fat content was analyzed by the Soxhlet extraction method with absolute ether as solvent following the methods of Li [19]. The method was modified by our practice experience. The detailed steps are described as follows:

1) The filter paper was folded to make a bag and the bag was air-dried for 2 h in an oven at 105˚C. After drying, the filter paper was cooled to room temperature in desiccators. About 30 min later, the filter paper was weighed in an analytical balance, and the weight was noted as W_0.
2) 1 g seeds were ground and loaded into the filter paper bag. Then the bag was weighed in an analytical balance, and the weight was noted as W_1.
3) The bags were put into the Soxhlet extractor with absolute ether for 8h. After that, the paper bag was air-dried for 2 h in an oven at 105˚C and was weighed in an analytical balance, and the weight was noted as W_2.
4) Crude fat content = $(W_1 - W_2)/(W_1 - W_0) \times 100\%$.

2.8. Analysis for Lipase

Lipase activity was analyzed by basic titration. 3 g seeds were homogenized with 5 ml 0.1 mol·L^{-1} of Phosphate-citrate buffer, the homogenate was used for lipase assay. The detailed steps were described as follows **Table 2**.

Then use 0.1 mol·L^{-1} of NaOH to titrate the solution to reddish color.

3. Results

3.1. Seed Germination

As shown in **Figure 1**. Seed germination was affected by cold stratification. Seeds started to germinate when they were stratified for 90days. And the finial germination of seeds with no GA_3 treatment was 12.7%. In contrast, GA_3 significantly increased seed

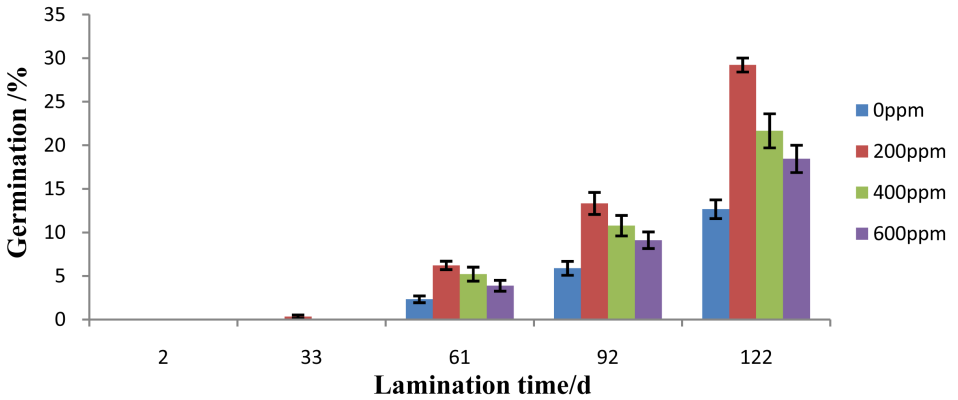

Figure 1. Germination (%) of *Cephalotaxus sinensis* seeds treated with different concentrations of exogenous GA_3.

Table 2. The reagent content and operating sequence of enzyme activity assay.

Action item	Number		
	A1	A2	A3
Distilled water/ml	5	5	5
Triolein/ml	1	1	1
shake shock/min	1	1	1
Heat preservation (100˚C)/min	5	0	0
Toluene/drop	5	5	5
Heat preservation (37˚C)/h	24	24	24
Brown-Brenn (4:1)/ml	50	50	50
Phenolphthalein/drop	4	4	4

germination depending on concentration. At the concentration of 200, 400, 600 mg/L, seed had reached a germination to 29.2%, 21.7%, and 18.4%, increasing by 16.5%, 9%, and 5.7% compared to those without GA_3 treatment, respectively. Obviously, the germination of seeds treated with 200 mg/L GA_3 was higher than others, which explaining that cold stratification with 200 mg /L GA_3 treatment was an effective method to break seed dormancy.

3.2. Soluble Sugar and Starch

The results of soluble sugar analysis of seeds during cold stratification are presented in Figure 2(a). During the low temperature treatment period from beginning to 45 days, the soluble sugar content in seeds treated with 0, 200, 400, 600 mg/L of GA_3 decreased gently from 2.35, 2.68, 2.60, 2.33 $mg \cdot g^{-1}$ to 1.38, 1.53, 1.42, and 1.27 $mg \cdot g^{-1}$, respectively. After that, soluble sugar content showed a sharp increase until 5 months lamination. At the end of cold stratification, soluble sugar content increased to 3.36, 3.69, 3.38, and 3.68 $mg \cdot g^{-1}$. On the contrary, the starch content decreased significantly (Figure 2(b)). During the low temperature treatment period, the starch content in seeds treated with

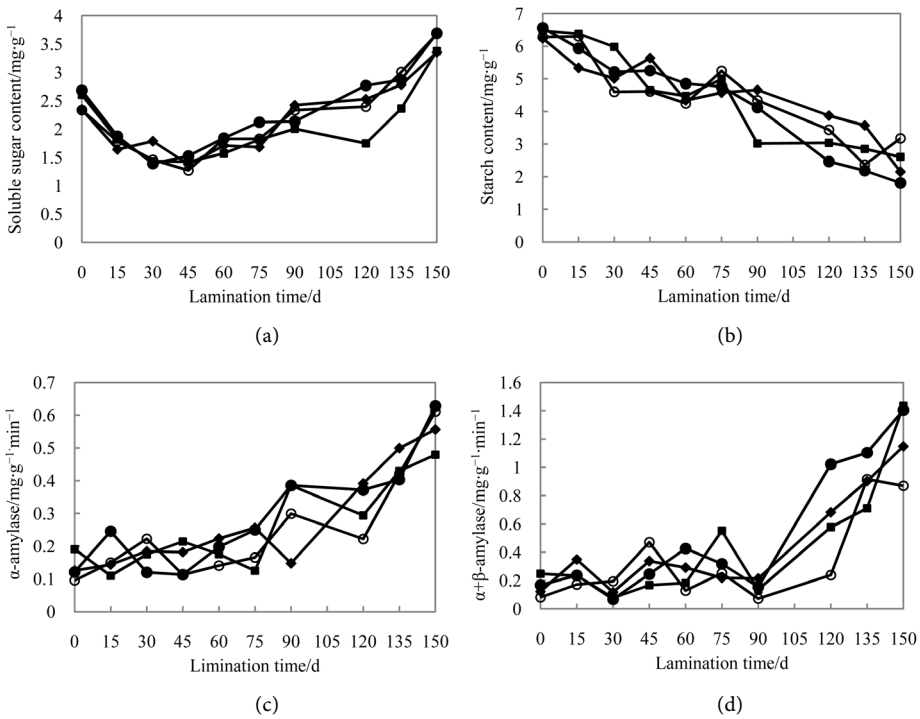

Figure 2. Effects of cold stratification on soluble sugar (a); starch (b); protein (c), free amino acid (d) content and α-amylase and $\alpha + \beta$-amylase activity in seeds treated with 0 (-◆-), 200 (-●-), 400 (-■-) and 600 (-⊖-) mg/L of GA_3.

0, 200, 400, 600 mg/L of GA_3decreased to 2.15, 1.81, 2.61, and 3.18 $mg \cdot g^{-1}$, decreased by 65.5%, 72.6%, 59.6%, and 49.4%, compared to those without cold stratification treatment, respectively.

3.3. Amylase Activity

The low temperature treatment showed significant effects on amylase activity. During cold stratification treatment period, both of α-amylase (**Figure 2 (c)**) and $\alpha + \beta$-amylase (**Figure 2(d)**) activity increased dramatically. The α-amylase activity in seeds without GA_3 treatment increased slowly until seeds were laminated for 90days, and then showed a rapid increase at 120, 135 and 150 days, corresponding to 0.391, 0.556, and 0.499 $mg \cdot g^{-1} \cdot min^{-1}$, respectively. The trends of other three treatments were the same as it. Similarly, $\alpha + \beta$-amylase activity showed a trend of rising as a whole, despite the fluctuation at the early stage of treatment. After 90 days lamination, $\alpha + \beta$-amylase activity in seeds without GA_3 treatment reached to 0.215 $mg \cdot g^{-1} \cdot min^{-1}$, increased by 70.8% compared to the control 0.123 $mg \cdot g^{-1} \cdot min^{-1}$. And the activity at 120, 135 and 150 days increased by 454.5%, 634.1%, and 834.1%, respectively. The maximum activity of $\alpha + \beta$-amylase appeared in seeds treated with 200mg/L of GA_3 and it increased to 1.149 $mg \cdot g^{-1} \cdot min^{-1}$ at the end of cold stratification treatment. Notably, there was no significant difference between α-amylase and $\alpha + \beta$-amylase activity during the treatment period from 0 to 90 days. After 90 days, the activity of $\alpha + \beta$-amylase was significantly higher than that of α-amylase during the same period of time.

3.4. Protein and Free Amino Acid

The results of protein content in seeds during cold stratification are presented in Figure 3(a). There were no obvious changes and differences of protein content in seeds with difference treatments in the early phase of cold stratification. During this time, protein content in seeds treated with 0, 200, 400, and 600 mg/L of GA_3 decreased by 8%, 11%, 4%, and 3%, respectively. As the cold stratification treatment duration increased, the protein content showed a sharp decrease after 75 days, and the most significant decrease in protein content was in 200 mg/L GA_3-treated seeds. In seeds stratified for 90, 120, and 150 days it decreased significantly to 16.4, 15.2, and 7.0 $mg \cdot g^{-1}$, with 8%, 11%, and 42% lower than that without GA_3 treatment, respectively. Conversely, free amino acid content showed a trend of rising as a whole in spite of slightly decreased during treatment period from 0 to 30 days (Figure 3(b)). The most significant increase in free amino acid content was in 200 mg/L GA_3-treated seeds.

3.5. Crude Fat and Lipase

The results of crude fat content in seeds during cold stratification are presented in Figure 4(a). As a result of low temperature stress, the crude fat content in both treatments increased first then decreased. During the low temperature treatment period (0 - 30 DAT), the crude fat content increased. The content in seeds treated with 200, 400 and

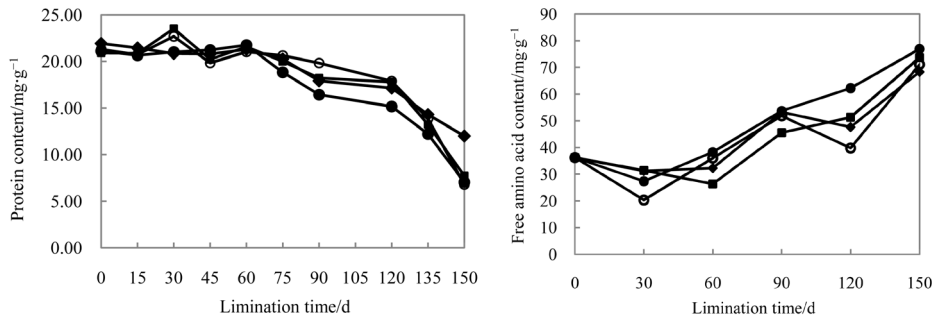

Figure 3. Effects of cold stratification on protein(a) and free amino acid (b) content in seeds treated with 0 (◆), 200 (●), 400 (■) and 600 (⊖) mg/L of GA_3.

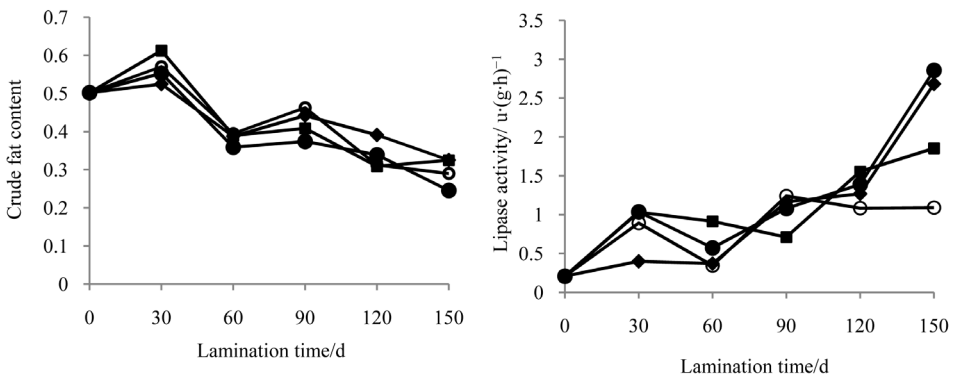

Figure 4. Effects of cold stratification on crude fat content (a) and lipase activity (b) in seeds treated with 0 (◆), 200 (●), 400 (■) and 600 (⊖) mg/L of GA_3.

600 mg·L^{-1} GA_3 was 55.28%, 61.22% and 57%, higher than that without GA_3 treatment, and the contents all reached the highest point. During the low temperature treatment period (30 - 150 DAT), crude fat content decreased significantly. After 150 days, crude fat contents in different treatments were 32.5%, 24.52%, 32.49% and 29%, respectively. The decrease of crude fat content indicated that crude fat was hydrolyzed to fatty acids and sugars to provide for seed germination.

As the cold treatment duration increased, the lipase activity in both treatments varied (**Figure 4(b)**). The lipase activity in seeds treated with 400 and 600 mg·L^{-1} GA_3 increased steadily. After 150 days, lipase activity was 1.85 and 1.09 u·(g·h)$^{-1}$. However, during the low temperature treatment period (0 - 120 DAT), lipase activity in seeds treated with 0, 200 mg·L^{-1} GA_3 increased slowly. Then it increased rapidly. After 150 days, lipase activity was 2.68 and 2.86 u·(g·h)$^{-1}$, higher significantly than other two treatments.

4. Discussion

For many plant species, seed dormancy can be broken by cold stratification. Recently, cold stratification has been widely used to break seed dormancy and enhance the germination percentage. In *A. tripolium*, the germination of seeds under cold treatment was obvious higher than that without stratification [20]. Also, Lohengrin *et al.* [21] indicated that seed germination with stratification increased significantly. In addition, some research indicated that exogenous GA can promote seed dormancy breaking process [8] [22]. In seeds of Peach and *Paris polyphylla* var. Yunnanensis, it has been found that synergistic effect between cold stratification and GA treatment played an important role in breaking seed dormancy [23] [24]. Seeds of *Cephalotaxus sinensis* remain deeply dormant in natural condition, and they will not germinate until the next or third year after landing. Consistent with this, Result of the present study showed that seed germination reached to 12.7% after stratified for 150 days, which indicating that cold stratification could break the dormancy and improve germination percents of *Cephalotaxus sinensis* seeds. Additionally, seeds with cold stratification and GA_3 treatment reached germinations to 29.2%, 21.7%, and 18.4%, much higher compared to the contrast. Therefore, cold stratification combined with GA_3 treatment could break seed dormancy and promote seed germination rates. And 200 mg/L of GA_3 was the best for *Cephalotaxus sinensis* seeds to release seed dormancy.

With seed germination, there will be some physiological and biochemical changes in seeds during cold stratification. The primary reserve materials, such as starch, protein, are important energy sources for seed germination [25]. And the rapid hydrolysis of seed reserves is essential for seed germination [26]. Once a seed starts to germinate, certain developmental events are initiated within the food storage tissues to ensure that reserves are hydrolyzed by hydrolytic enzymes to provide essential soluble products for seedling growth [27]. Many studies have shown that a series of conversion between primary reserves and essential soluble products occurred during cold stratification [9] [28]. In our study, the starch content decreased and amylase activity increased signifi-

cantly. It turned out that during treatment period, starch was hydrolyzed to soluble sugar by amylase, which was consistent with the increasing trend of sugar on the whole. However, soluble sugar content decreased first. A possible explanation for this effect is that seeds kept breathing throughout the lamination process, consuming some energy provided by soluble sugar. In the early stage of lamination, the content of sugar derived from starch hydrolysis was lower than that of sugar for respiration. This is consistent with Liao [29] and Wang [28] who showed that starch was hydrolyzed to soluble sugar and contents of the two were negatively correlated. Notably, the turning point of amylase activity trend occurred when seeds were stratified for 90 days, at which time seeds started to germinate. The results indicated that starch hydrolysis was mainly catalyzed by α-amylase in the early stage of lamination. And when seeds started to germination, $\alpha + \beta$-amylase played a leading role in starch hydrolysis. The result is similar with the study Chen [30] did on *Spuriopimpinellabrachyara* (Kom.) Kitag seed.

In the process of germination, protein performs the function of providing nitrogen nutrition, and provides material and energy source for the seed differentiation at the same time [11] [31]. In seeds of *M. domestica*, protease activity increased markedly during cold stratification, indicating solubilization of reserve protein [32]. Also, in Hawthorn seeds, cell respiration rate and soluble protein content were increased through stratification, but the contents of free amino acid were decreased [33]. Consistent with this, results of the present study showed that the content of protein decreased markedly during cold stratification, and an increase quantity of the free amino acid paralleled the increase in seed germinating ability. A possible explanation for this effect is that reserve protein was hydrolyzed to free amino acid by protease, meeting the needs of seed germination. This supports the ideas that indicated by Wang *et al.* [31] and Bewley *et al.* [10]. The results indicated that the reserve of starch and protein were important energy sources for seed germination and cold stratification could accelerate the mobilization of seed reserves. Consequently, cold stratification is an effective way to break seed dormancy.

Crude fat was one of the most important reserves. During the low temperature treatment period, crude fat was hydrolyzed to fatty acids and sugars by lipolytic enzymes [10]. As the cold treatment duration increased, the crude fat content in seeds of *Cyclocarya paliurus* showed an increasing trend and the lipase activity showed a decreasing trend [31]. Zhang *et al.* [34] pointed out that during cold stratification, crude fat content in seeds of *Taxus mairei* decreased by 40% than that without treatment. The decrease of crude fat was due to turning to soluble sugar. As the cold treatment duration increased, crude fat content decreased and lipase activity increase significantly, indicating that crude fat was hydrolyzed into sugar and fatty acids by lipase. These results were in agreement with the findings of Gao *et al.* [35] and Huang *et al.* [36].

5. Conclusion

In brief, cold stratification and exogenous GA_3 are required for dormancy breaking and germination of *Cephalotaxus sinensis* seeds. During this time, a series of biochemical

changes occur in seeds. For example, starch and proteins were hydrolyzed, resulting in changes in soluble sugar and free amino acid to provide energy for seed germination. The decomposition, transformation and utilization of storage substance, such as starch, protein, may be the key breaking seed dormancy.

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