**The causes of European hornbeam seed dormancy and methods of breaking dormancy**

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\*Corresponding author. Tel:0086-13002509011. Email: zhuzunling@yahoo.com.cn.ABSTRACT: A systematic study was conducted to explore the causes of seed dormancy in European hornbeam(*Carpinus betulus*). European hornbeamseeds undergo combinational dormancy: the mature seed has a complete configuration, but the embryo requires after-ripening, which is the main cause of dormancy. The seed coat of the European hornbeam seeds is very hard and dense, which hinders water absorption to some degree. The seed coat also contains some germination inhibitors. We found that soaking the seeds in 500 mg L-1 GA3 could increase the germination rate and that concentrated sulfuric acid scarification could significantly reduce the time of stratification.

KEY WORDS: *Carpinus betulus*, Seed, Dormancy, Lifting method

**1 Introduction**

Seed dormancy is a very complex process, and dormancy has many possible causes. The unique ultra-microstructure and characteristics of some seeds, such as a compact wax coat and sophisticated covered cuticles on the testa of a seed, suberification of the testa cellulose and pectin and osteosclereide formed in collenchymatous cells, can result in poor air and water permeability and even mechanical restraint by the seed coat that can block germination. The seeds of Leguminosae, Polygonaceae, Sterculiaceae and Tiliaceae always exhibit seed dormancy (Bewley & Black 1994). Poor air and water permeability of the seed coat or pericarp is an important reason for the dormancy of some seeds, such as *Davidia involucrate* (Wan & Anatomical 1988), *Taxus chinensis* var. mairei (Zhang 2007;Shi 1991) and *Gleditsia sinensis* (Zhang et al. 2004). Some seeds have embryo dormancy (Bewley & Black 1989)in which the dormancy is inherent in the mature embryo rather than being imposed by the seed coat; examples include *Taxus baccata*, *Malus sylvestris* and *Taxus baccata* var. fastigiata. Embryo dormancy can be divided into two forms: morphological and physiological (Leadem 1997; Hilhorst 1995). The physiological factors causing embryo dormancy include an immature embryo and the presence of inhibiting chemicals in such seed structures as the pericarp, seed coat, endosperm and embryo. In 1922, Oppenheimer found that tomato (*Lycopersicon esculentum*) seeds have poor permeability, however the main reason for seed dormancy was found to be the presence of an inhibitor (Oppenheimer et al. 1922). Proposing that inhibitors are one of the most important causes of dormancy (Kentzer 1966;Amen 1986),Kentzen and Amen separated inhibitors from the pericarp, seed coat, endosperm and embryo of some plants. Physiological suppression is caused in some seeds by such chemicals as fermentation products, hormones, soluble metabolites and other compounds; these inhibitors slow embryo growth such that the demands of germination cannot be met, thus causing dormancy. The genotype, environment of maturation and storage conditions also influence seed dormancy.

The factors affecting dormancy are not isolated and impact seed dormancy in combination, and the mechanisms involved in dormancy are complex (Chen et al. 1997). In most plants, dormancy is the result of many factors. Lipe and Crane suggested that mechanical resistance and internal inhibitors both prevent the germination of peach (*Amygdalus persica* Linn.) seeds (Lipe & Crane 1966).Germination in *Symplocos tetagona* (Xu et al. 2002)is constrained by its seed coat and by physiological dormancy, and *Sinojackia xylocarpa* (Shi et al. 1999)has a similar mechanism and requires low temperature stratification to germinate. *Schima superb* (Li 2004)possesses chemical inhibitors of germination and lacks germination enhancers, two factors that impact seed germination.

European hornbeam is a tree belonging to the Carpinus genus in family Betulaceae. Previous research suggests that European hornbeamhas deep dormancy habits and requires thermoregulation to break seed dormancy, and the International Seed Testing Association recommends breaking dormancy as follows: in a moist environment at 20˚C for 1 month, followed by 3~5˚C for 4 months (Paula & Carpinus 2008). However, seed dormancy causes many problems with the breeding of European hornbeam, and the dormancy mechanism of European hornbeamseeds remains unclear to date, and a rapid method for dormancy release has not been found. Accordingly, European hornbeam seeds were used to evaluate the water permeability of the seed coat, percentage of seed germination and presence of inhibitory substances. The reasons for dormancy and useful methods for breaking dormancy are also discussed. The results of this study can provide a theoretical and practical basis for a dormancy release mechanism forEuropean hornbeam seeds and for the large-scale introduction of such a technique in the near future.

**2** **Materials and methods**

**2.1** **Test material**

The test materials consisted ofEuropean hornbeamseeds imported from Poland (collected in October 2010) by the China National Tree Seed Corporation. The seeds were cleaned, naturally dried and then stored at 3~5˚C. Chinese cabbage (*Brassica chinensis*) seeds, known as “shanghaiqing” in the market, with a pureness of 96%, neatness of 98%, germination rate over 85% and a moisture content of approximately 7%, were also used.

**2.2** **Test method**

**2.2.1 Water permeability of the seed coat**

Scarified seeds (whole seeds for which the seed coat was cut), whole seeds (full and with a complete seed coat) and empty seeds (screened by X-ray), with 50 seeds in each group, were evaluated with 3 repetitions. The dry weight of each group was measured, and the seeds were placed in beakers with distilled water at a constant temperature of 25˚C. The seeds were removed from the water every 3 h, blotted with filter paper and re-weighed using an FA1004N electronic balance until they reached a constant weight. The water absorption was then measured as the difference between the dry and final weights; there were 3 repetitions for each, and the average value was obtained as a measure of the perviousness of the seed coat.



**2.2.2 Seed extracts of various parts of bioassay**

Approximately 200 fresh and full seeds were randomly selected. The skin and embryo were separated, attrited and placed in a 100 ml conical flask; 50 ml of 80% methanol solution was added, and the flasks were stored in the refrigerator at 0-4˚C under isothermal conditions for a 72 h closed extraction. The seeds were shocked after a certain time interval to ensure a full extraction. The experiment was repeated twice. The filtrate was dried at 35˚C using a vacuum rotary evaporator apparatus, rinsed with distilled water and obtained a 50 ml subsample of the filtrate. Methanol extracts of the various parts of the European hornbeam seeds were obtained. Then, the extracts were diluted to 25%, 50% and 75% of the original concentration (25% extracted fluid: the extracted solution was diluted to 25% of the concentration of the original solution; the 50% and 75% concentrations were prepared the same way).

The activity of chemical inhibitors in the hornbeam seeds was tested by assaying the effect of extracts on the germination of Chinese cabbage rapeseeds. The same volume of distilled water was used as a control. A 5 ml aliquot of different concentrations of the seed extract was applied to filter paper placed in 12 cm Petri dishes. The germination test was performed in a 25˚C constant-temperature illuminated incubator. The germination rate was recorded each day. The statistics on the germination rate (with a visible cotyledon being the germination standard) were calculated after 48 h, and the seedling height and root length were measured after 72 h. The germination test for each concentration of extract was repeated three times.

**2.2.3 Test for seed embryo dormancy characteristics**

Fresh and full European hornbeamseeds were selected, and the seed husks were removed under sterile conditions. The seeds were placed in germination boxes containing pledget moistened with distilled water. The boxes were placed in a 25˚C, 12 h light regime climate chamber to observe seed germination. The moisture and temperature of the germination environment were checked every day. Slightly musty seeds were removed, rinsed with water and then returned to the germination box. Germination beds were replaced timely when mold rot tablets increased. If no germination occurred after 30 days, the seeds were considered to be dormant.

**2.2.4 Method for breaking seed dormancy**

The seeds were etched with sulfuric acid (specific gravity 1.84 g cm-3) for 5 min and immediately rinsed in running water for 24 h. The seeds were then soaked with 500 mg L-1 GA3. The seeds were stratified in two different substrates: one of wet sand mixed with clear water (Ⅰ) and another of wet sand mixed with 500 mg L-1 GA3 (Ⅱ). The seeds were placed in artificial climate boxes for temperature-accelerated stratification after dressing the seeds. The seeds were first maintained at a constant temperature of 23˚C for 30 days and then at a constant temperature of 5˚C for up to 4 months of cold stratification. The seeds were assessed at 60 d, 90 d, 105 d, 120 d, 135 d and 150 d. Each treatment is summarized in Table 1.

Table 1 The comprehensive treatment of European hornbeam seeds

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Treatment Number** | **Concentrated Sulfuric Acid Treatment (min)** | **Concentration of GA3 (mg•l-1)** | **GA3** **Treatment Time (h)** | **Stratification Matrix** |
| A0 | 0 | 0 | 24 | I |
| A1 | 0 | 0 | 24 | II |
| B0 | 5 | 0 | 24 | I |
| B1 | 5 | 500 | 24 | I |
| B2 | 5 | 500 | 24 | II |

**2.3 Data analysis**

Microsoft Excel 2003 and SPSS 13.0 software were used for the data processing and statistical analysis.

**3 Results and analysis**

**3.1 Reasons for European hornbeam seed dormancy**

**3.1.1 Seed coat permeability**

Water absorption of the empty, scarified and whole seeds was measured. As shown in Figure 1, the water absorption of European hornbeam gradually increases with increasing water absorption time. The water absorption process of European hornbeam seeds can be divided into three periods: rapid absorption (0-9 h), slow absorption (9-60 h) and an almost saturated stage. The intact seeds complete 91.4% of the total water absorption by the end of the rapid absorption period, and the water absorption reaches 34.69% in the nearly saturated period. The variation in the water absorption curves of the intact seeds and scarified seeds is similar, but the water absorption of the whole seeds is lower than that of the scarified seeds. The difference between the scarified seeds and whole seeds is not significant, but the difference between the empty seeds and other two types of seeds is significant based on an analysis of variance and a multiple comparison of the water absorption of different types of European hornbeam seeds. Thus, we conclude that European hornbeam seeds have a certain amount of water permeability and that the seed coat is not the main inhibitor of seed germination.

**3.2.2 Bioassay of European hornbeam seeds**

The dormancy of many seeds is due to the presence of endogenous inhibitors, and, in general, methanol extracts of parts of the seed can reveal whether a seed contains endogenous inhibitors. The influence of the extract of different parts and extract concentrations on the germination rate of Chinese cabbage rapeseeds is shown in Figure 2, with the extract from different parts of the seed having different effects on the Chinese cabbage seed germination rate. The germination rate was 98% for the treatment of 100% seed embryo methanol extract, whereas the germination rates were 90%, 79% and 67% for the treatments of 50%, 75% and 100% seed coat methanol extract, decreases of 10%, 21% and 33%, respectively. Thus, the germination rate of Chinese cabbage rapeseeds treated with the seed coat methanol extract of European hornbeam tended to gradually decrease as the concentration increased. The difference in the germination rates of Chinese cabbage rapeseeds treated with different concentrations of extract is significant based on the analysis of variance and Duncan’s multiple comparison. The European hornbeam seeds coat extract solutions at 25% did not show significant differences compared to the control; the other treatments for the germination of Chinese cabbage rapeseeds were all significantly different from the control.

**3.3 Seed embryo dormancy test**

The European hornbeam in vitro embryos failed to germinate within 30 days, indicating that the seed embryo itself exhibits certain dormancy characteristics and may only be able to germinate following morphological and physiological after-ripening.

**3.4 Effects of the different methods of overcoming dormancy on the seed germination of European hornbeam**

The seed germination rate of European hornbeam in response to different methods of overcoming dormancy is shown in Figure 3. A0 indicates the temperature-accelerated stratification process, and only a few seeds had germinated after 60 d, 90 d, 105 d and 135 d. However, the germination rate increased greatly after 150 d. Treatment A1, in which seeds were soaked with 500 mg L-1 GA3, was better than separate stratification; after the sulfuric acid scarification, treatments B0, B1 and B2 resulted in different degrees of mildewed and decaying seeds in the late stratification period. It is possible that the sulfuric acid used was harmful to the seeds. However, the germination time of seed treatment B2 was faster than the other treatments, clearly indicating that concentrated sulfuric acid scarification and 500 mg L-1 GA3 could significantly reduce the time of stratification.

**4 Conclusions and discussion**

Although the structure of the European hornbeam seed embryo was clear, it failed to germinate through embryo culture in vitro. The seed embryo required after-ripening and was the main cause of dormancy. The seed coat of the European hornbeam was keratinized and, therefore, hard and dense. We found that the water absorption trends of the intact seeds and scarified seeds were similar but that the saturation point of the scarified seeds was higher than that of the intact seeds; these results indicated that the seed coat hindered water absorption to some degree, though the difference was not significant. Over the course of the experiment, the color of the seed kernel changed from weathered white to milky white; it was therefore clear that the seed was water permeable to some degree and that the water permeability of the European hornbeam seeds was not the main reason for seed dormancy in this species. The bioassay of European hornbeam seeds indicated that the seed coat of European hornbeam contained a substance that was inhibitory to the germination of *Brassica campestris* seeds. Although the effect on the germination rate was not obvious when the methanol extract concentration was low, the inhibitor content increased with an increase in the methanol extract concentration, and the resulting effect on the *Brassica campestris* seed germination rate was notable.

Based on our results, we can conclude that the dormancy of European hornbeam seeds can be characterized as combinational dormancy. The matured seed of European hornbeam has a complete configuration, but its embryo requires after-ripening, which is the main cause of dormancy. The seed coat of European hornbeam seeds is very hard and dense, which hinders water absorption to some degree. The seed coat also contains some chemical germination inhibitors, and these factors are additional important causes of seed dormancy.

The germination test of European hornbeam seeds resulted in the finding that the dormancy of European hornbeam seeds can be effectively overcome by alternative stratification. Soaking the seeds in 500 mg L-1 GA3 could heighten the germination rate, however, this treatment cannot effectively reduce the time of stratification. The optimal concentration of GA3 needs be further studied. Although concentrated sulfuric acid scarification could significantly reduce the time of stratification, the cost of seed lost to rotting was significant. Therefore, the suitable concentration and treatment time of acid scarification need to be further studied to identify the most effective way to break seed dormancy in this species.

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Figure 1 Water absorption of European hornbeamseeds

Figure 2 The germination rate of *Brassica campestris* after treatment with different concentrations of extract from European hornbeam seeds

Figure 3 Effects of the study treatments on the germination percentage of European hornbeam seeds

Note: Duncan’s method for multiple comparisons. Different uppercase letters indicate a significant difference at P <0.01; different lowercase letters indicate a significant difference at P <0.05; the same lowercase letter indicates no significant difference (P> 0. 05).