Introduction

Coix lachrymal-jobi L, an annual or perennial Graminea herb, is an important food and drug homologous plant rich in nutrition and excellent for disease prevention and health care, and it is of extensive application. However, due to its past inadequate research, the cultivation area has been on a sharp drop and its wild resources has been greatly reduced. In recent years, with the increasing of people’s awareness for health care, increasing studies have been conducted on its cultivation extension, tissue culture and rapid propagation, germplasm resource protection, and food and drug interaction (Li et al. 2016).

Anther culture is a type of tissue culture in which anthers at appropriate development stage are inoculated on artificial medium to induce the differentiation and mitotic division to form cell masses, so as to form undifferentiated calluses, or to differentiate into embryos, and then induce the calluses to differentiate into complete plantlets. Anther culture is an effective way to obtain homozygote haploid, which can effectively shorten the breeding period, overcome the incompatibility of distant hybridization, and obtain excellent traits. The application of anther culture has been extensive and breakthroughs have been made in tobacco, rice, corn, oilseed rape and other crops (Jiradej et al. 2014). A few studies on in vitro germination and culture of the anther of Coix have been documented. Early in vitro anther culture studies of Coix by Li et al (1981) showed that the anther of Coix was most suitable for growing on H medium and regenerating haploid plants. Wang et al (1980) studied the induction of sporophyte from Coix lachrymal-jobi L pollen using N6 culture medium, and obtained callus and embryoid, and found that the plants formed from calluses contain the haploids, diploids and mixoploids, and the plants formed from embryoids showed more stable haploid, but the seedling rate was low. Li et al (1997) observed the meiosis process of pollen mother cells of Coix and recorded the meiosis process in detail. Dong and Xi (1992) recorded in detail the mega- and micro-sporogenesis and the formation of male and female gametophytes in Coix lachrymal-jobi L.

In this research, by using the Coix cv GDYY, pollen microspore morphology at different flowering stages were observed, in vitro pollen germination conditions were identified, anthers were cultured, and pollen vigor of 7 Coix cv were determined, in order to lay a foundation for further research on haploid breeding of Coix lachrymal-jobi L by anther culture.

Materials and Methods

Materials

Coix lachrymal-jobi L cv. Seeds of the 7 Coix lachrymal-jobi L cv (Table 1) were harvested in Fairy Mountain Town, Wulong District, Chongqing, China.

Methods

Morphological observation on flowers and pollen grains of Coix cv: Judging from the appearance of male flowers, the
flowing of *Coix lachrymal-jobi* L occurs in the early July, and the male flowers became full open in about 7~11 days and would last for about 3~7days. To observe the flowering characteristics of *Coix lachrymal-jobi* L, the flowering period is divided into the early, peak and late flowering stages mainly based on the appearance of male inflorescence and the male inflorescence were picked at 9:00-10:00 am.

The characteristics of the male spikes (male inflorescence), male spikelets (flower buds), pollen were observed and compared. Male inflorescence were fixed with Carnoy’s fluid fixative for 24h, transferred to 70% ethanol, and stored in 0 ~ 4℃ in refrigerator. The mid-region male spikelets of the male inflorescence were placed on the glass slide, cut open with a scalpel, squeezed out the pollen with tweezers, and dyed with I₂-KI. Then microscopic observation were carried out.

**Determination of pollen vigor at different flowering stages:**
Pollen vigor was determined by I₂-KI staining. Anthers of the 3 observation were carried out.

**Determination of optimum time.** Pollen of *Coix cv GDYY* were cultured in the above-determined optimal medium and temperature, and the pollen germination rates over time were estimated to determine the optimal germination time.

**Observation and identification of microspore development phases:** Male inflorescence of *Coix cv GDYY* at the early flowering stage was pick, and the longitudinal lengths of the inflorescence, bud, and anther in the middle were measured. The microscopic characteristics of the microspores were observed after Carnoy fixation of the anthers for about 20min, and then 70% ethanol preservation. Finally smear slides were made and stained by I₂-KI for microscopic determination of the phases of microspore development.

**Anther treatment and culture conditions:** For there are some correlation between pollen development phase and some external morphological features of the flower bud, it is possible to use these external markers to select the flower buds that is close to the required phase. During the experiment, one anther from each bud should be taken to determine the phase of pollen development through microscopic examination.

According to Li (1981), anther treatment and culture conditions are different from those of the vegetative tissues or organs. The flower buds with microspore development mainly in the uninucleate marginal phase were pick and sterilized by washing with 75% alcohol for 1 min, then rinsing 3 times with sterile water, sterilizing with 0.1% HgCl₂ for 5 min, and rinsing 5-8 times with sterile water, and finally dried with sterile dry filter paper. Anthers from the sterilized flower buds were pick and cultured in MS medium with 30 g/L sucrose and 6 g/L agar (pH 5.6-5.8) at 30 ℃ and in dark for 20 days and then transferred to 25 ℃ light 12h (2000lx) for 10 days (Dong et al., 2017).

**Callus and embryoid induction, multiplication and seedling differentiation:** By reference to the report of Gao et al. (2005), 6 callus induction media were prepared: ① MS + 2, 4-D 1 mg/L + KT 1 mg/L, ② MS + 2, 4-D 1 mg/L + KT 1.5 mg/L, ③ MS + 2, 4-D 1 mg/L + KT 2 mg/L, ④ MS + 2, 4-D 2 mg/L + KT 1 mg/L, ⑤ MS + 2, 4-D 2 mg/L + KT 1.5 mg/L, and ⑥ MS + 2, 4-D 2 mg/L + KT 2 mg/L. To each medium 2 mg/L AgNO₃ was added. After 5 days of anther culture, the callus induction was observed and the rate of callus formation was estimated, in order to find the optimal medium for callus induction.

After culture for one month, calluses were transferred to multiplication medium ⑦ (MS + 2, 4-D 2 mg/L + KT 1.5 mg/L + GA 1 mg/L). The multiplied calluses was inoculated into the differentiation media ⑧ (MS + IAA 0.5 mg/L + KT 1 mg/L), ⑨ (MS + IAA 0.5 mg/L + KT 2 mg/L), and ⑩ (MS + IAA 0.5 mg/L + KT 2.5 mg/L). Record the seedling differentiation, in hope to find the optimal differentiation medium for seedling differentiation.

**Results**

**Morphological observation of Coix pollen grains at different flowering stages**

The male tassels, female panicles and pollen grains of *Coix lachrymal-jobi* L showed different morphological characteristics.

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**Table 1: Coix lachrymal-jobi L CV Used in This Study.**

<table>
<thead>
<tr>
<th>CV</th>
<th>CV Code</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guangdong Yi Yi</td>
<td>GDYY</td>
<td>Guangzhou, Guangdong, China</td>
</tr>
<tr>
<td>Xing Ren Xiao Bai Ke</td>
<td>XRXBK</td>
<td>Xingyi, Guizhou, China</td>
</tr>
<tr>
<td>Guangxi Yi Yi</td>
<td>GXYY</td>
<td>Xulin, Guangxi, China</td>
</tr>
<tr>
<td>Fujian Yi Yi</td>
<td>FYY</td>
<td>Pucheng, Fujian, China</td>
</tr>
<tr>
<td>Hebei Yi Yi</td>
<td>HBYY</td>
<td>Anguo, Hebei, China</td>
</tr>
<tr>
<td>Zhe Yi Yi Hao</td>
<td>ZYH</td>
<td>Lishui, Zhejiang, China</td>
</tr>
<tr>
<td>Liaoning Bai Ke</td>
<td>LNBK</td>
<td>Jinzhou, Liaoning, China</td>
</tr>
</tbody>
</table>
in the early, peak and late flowering stages, as exemplified by the GDYY cv (Figure 1). In the early flowering stage, the male tassels were green, ca. 18~21 mm in length, male flowers were not yet in bloom, while the stigmas were extending out fresh and tender, clearly indicating the asynchronous development of the male and female flowers, i.e. the female matures ahead of the male flowers. The caryopses were dark green (Figure 1A). After I₂-KI staining, it was observed that about 60% of the pollen grains were dyed light yellow or brown and about 40% were dyed blue; and the size of the spherical pollen grains varied. Microspores of uni- and bi-nucleate phase coexisted with one germination pore (Figure 1B, C). These clearly indicated the immature male flowers at this early flowering stage. The male tassels at the peak flowering stage were yellow green, ca. 21~25 mm in length, the stigmas mostly withered, while the male flowers were in full bloom, and the yellow tender anthers fully exposed, ready for pollinating; the caryopses were yellow green (Figure 1D). Almost all the pollen grains at this stage were uniformly spherical, in bigger size and dyed dark blue (Figure 1E, F), indicating the full maturation of the male flowers. At the late flowering stage, tassels were deep yellow, ca. 22~25 mm in length, the male spikes were almost die-out, anthers turned brown, the stigmas completely died-out and fell off, and the caryopses were yellow (Figure 1G). About 60% of the pollen grains were normal in size and shape, and dyed blue, and about 40% irregularly shrunken and dyed yellow (Figure 1H, I).

**Observation of the pollen microspore development stages**

Microscopic observation of the microspores in anthers of *Coix lachrymal-jobi* L in the early flowering stage showed continuous development, with the late uni-, bi- and tri-nucleate phases coexist, as shown here in GDYY (Figure 2 A-C). But it is not synchronous since different phases of microspores occurred in the same piece of anther (Figure 2 D).

In order to identify anthers in the late uninucleate phase for later anther culture, the morphological characteristics of

![Figure 1](image1.png)

**Figure 1** I₂-KI Stained Pollen Grains of Coix Exemplified by CV GDYY at Different Flowering Stages.

A, D and G are the morphology of *Coix lachrymal-jobi* L in the early, peak and late flowering stages respectively; B, E and H are pollen micrographs (20×) from early, peak and late flowering stages respectively; and C, F and I are pollen micrographs (100×) from the early, peak and late flowering stages respectively.
flowers when the uninucleate microspores accounted for more than 60% were observed to be in the early flowering stage, at which the male panicle was compact and 1.8-2.1 cm in length, the longitudinal length of the flower bud was 0.8-1.0 cm, the anthers were light yellow and 0.4-0.5 cm in length; stigmas were fresh, 1.2-1.6 cm in length, the caryopses were dark green. At this stage the pollen was spherical and only had small vacuoles.

Comparison of pollen vigor of different Coix cv at different flowering stages

Microscopic observation and statistical data showed that pollen vigor varies greatly in different flowering stages and among different cv (Table 2). All the 7 cv had the greatest vigor in the peak flowering stage, followed by the late flowering stage, while the lowest had been in the early flowering stage due to the less starch accumulation in this immature stage. Pollen vigor of different cv was significantly different in the same flowering stage. At the peak flowering stage the pollen vigor of all the cv was well-above 85%.

Optimal conditions for pollen germination in vitro

Optimal pollen germination liquid medium: Pollen from different Coix cv at the peak flowering stage was cultured in boric acid (0.01%) solution containing 5%, 10%, 15% and 20% sucrose respectively for 5h and the germination rate was estimated. There were significant differences in germination rate and optimal germination medium for the 7 Coix cv (Table 3). The germination rates of GDYY, GXYY and HBYY were all above 50% in 15% sucrose +0.01% boric solution, the highest being 53.7% for GDYY, and thus the optimal culture solution for GDYY, GXYY

Table 2: Comparison of Pollen Vigor of Different Coix CV at Different Flowering Stages.

<table>
<thead>
<tr>
<th>Coix cv code</th>
<th>Pollen vigor at different flowering stages (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Early flowering stage</td>
</tr>
<tr>
<td>GDYY</td>
<td>29.9±2.7a</td>
</tr>
<tr>
<td>XRXBK</td>
<td>17.2±3.4de</td>
</tr>
<tr>
<td>GXYY</td>
<td>13.9±0.8e</td>
</tr>
<tr>
<td>FJYY</td>
<td>26.3±4.1ab</td>
</tr>
<tr>
<td>HBYY</td>
<td>29.9±3.6a</td>
</tr>
<tr>
<td>ZYYH</td>
<td>24.5±1.5abc</td>
</tr>
<tr>
<td>LNBK</td>
<td>15.8±1.7de</td>
</tr>
</tbody>
</table>

Note: data are shown as Mean ± SD (n=3); data in the same column with different lowercase letters are significantly different (P < 0.05)

Table 3: Effect of Different Concentrations of Sucrose in 0.01% Boric Acid Solution on Pollen Germination Rate of Different Coix CV.

<table>
<thead>
<tr>
<th>Coix cv code</th>
<th>Sucrose concentration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td>GDYY</td>
<td>27.4±6.7aC</td>
</tr>
<tr>
<td>XRXBK</td>
<td>9.5±0.9efC</td>
</tr>
<tr>
<td>GXYY</td>
<td>18.4±1.1bcC</td>
</tr>
<tr>
<td>FJYY</td>
<td>15.3±1.9cdBC</td>
</tr>
<tr>
<td>HBYY</td>
<td>15.2±1.6cdC</td>
</tr>
<tr>
<td>ZYYH</td>
<td>22.4±2.5abC</td>
</tr>
<tr>
<td>LNBK</td>
<td>13.8±1.3odeC</td>
</tr>
</tbody>
</table>

Note: data are shown as Mean ± SD (n=3); data in the same column with different lowercase letters are significantly different (P < 0.05), and data in the same row with different uppercase letters are significantly different (P < 0.01)
and HBYY was 15% sucrose + 0.01% boric acid. The germination rates of the other 4 cv, XRXBK, FJYY, ZYYH and LNBK were the highest in 10% sucrose + 0.01% boric acid, being the optimal medium. The pollen germination rates of the 7 cv were the lowest in the 20% sucrose + 0.01% boric acid solution.

**Optimal pollen germination temperature:** Pollen from GDYY, GXYY and HBYY at the peak flowering stage was cultured in the optimal medium of 15% sucrose + 0.01% boric acid, and XRXBK, FJYY, ZYYH and LNBK in the optimal medium of 10% sucrose + 0.01% boric acid at 20, 25 and 30℃ respectively for 5h and pollen germination rates were estimated (Table 4). It is evident that the optimal temperature for pollen germination is 25 ℃ for all the *Coix* cv.

**Optimal pollen germination time:** Continuous microscopic observation showed that the *Coix* pollen germination occurred slowly. Germination increased over the time of incubation with the 5h germination rate being the highest, around 50%. And after 5h, the pollen germination rate did not increase significantly. The extension of pollen tube also slow, and pollen tubes entangled after 15h when the pollen germination rate was not convenient to estimate. Therefore, 5~7h is the most appropriate time for estimation of pollen germination rate (as exemplified by GDYY in Figure 3). Only one circular germination hole and single-tube germination were observed for *Coix* pollen. And the blue-dyed pollen grain germinated readily and their pollen tubes grew faster.

**Callus, embryoid induction and differentiation process in *Coix* anther culture**

Anthers in the uninucleate phase at the early flowering stage were freshly pick, surface-sterilized, and inoculated for aseptic culture on MS medium. It was observed that when cultured for 10 days tender-yellow to white lump materials began to outgrow from one end while the other end seemingly dehydrated and somewhat shriveled. The opalescent lump materials were calluses or embryoids (Huang et al., 2000), and if the anthers turned brown in culture, it often failed to induce. The calluses or embryoids expanded then and reached maximum after about 30-40 days of culture. Embryoids were different in shape from calluses. Calluses were often loose, amorphous, crystalline and transparent, and could be further propagated and differentiated

| Table 4: *Coix lachrymal-jobi* L. Pollen Germination Rates at Different Temperatures. |
|-----------------------------------------------|----------------|----------------|----------------|
| *Coix* cv code | 20℃ | 25℃ | 30℃ |
| GDYY* | 18.7±4.9c | 44.1±2.4a | 33.8±2.0b |
| XRXBK | 3.8±0.7c | 24.0±1.2a | 18.2±1.7b |
| GXYY* | 14.2±2.7b | 44.3±2.3a | 43.4±1.8a |
| FJYY | 8.9±0.6c | 41.8±1.7a | 30.1±1.3b |
| HBYY* | 13.9±1.2c | 40.4±3.5a | 34.5±0.9b |
| ZYYH | 7.6±0.5b | 38.9±1.7a | 36.9±2.3a |
| LNBK | 5.9±0.8c | 36.2±1.2a | 17.9±1.6b |

Note: *: determined in optimal medium of 15% sucrose + 0.01% boric acid and the others in 10% sucrose +0.01% boric acid; data are shown as Mean ± SD (n=3); data in the same row with different lowercase letters are significantly different (P < 0.05).

Figure 3 Germination Process of *Coix* Pollen over Time. Exemplified by GDYY.
into cluster buds on the differentiation medium; embryos were compact, less transparent, typical shape of zygotic embryos and generally ceased to grow up to a certain size. Usually only one plantlet was differentiated from one embryoid, as exemplified by GDYY (Figure 4).

**Callus and embryoid induction rate in different media**

Effects of different media on callus and embryoids formation from anther culture of different *Coix cv* were studied. The callus and embryoid induction rates of different *cv* were significantly different (Table 5), among which *Coix cv* GDYY, GXYY, HBYY and LNBK had higher induction rates, up to 20%. And the same *cv* had significant difference in induction rate among the 6 media. Relatively speaking, for each *cv*, media ② and ⑤ had the higher induction rates. So the optimal media for induction of callus and embryoid were MS + 2, 4-D 1~2 mg/L + KT 1.5 mg/L. It was also observed that GDYY, GXYY and HBYY with higher pollen vigor as determined previously (See Table 2) have higher rate of callus and embryoid induction (Table 5).

It was observed that anthers of all the 7 tested *cv* could be effectively induced to form calluses and embryoids in the optimum media of MS + 2, 4-D 1~2 mg/L + KT 1.5 mg/L. In the differentiation medium ③ (MS + IAA 0.5 mg/L + KT 2 mg/L), a few inducers could be differentiated into small clumped buds or seedlings, but the seedling formation rate was very low.

**Discussion**

In this study, the morphology of flowers and pollen grains at different flowering stages of *Coix cv* were observed for the first time. After I<sub>2</sub>-KI staining, most of the pollen grains at the early flowering stage were stained light yellow or brown and were heterogeneous in size, and showed irregular shapes ranging from ellipsoidal or spherical with distinct uninucleate or binucleate phases and single germination pores. At the peak flowering stage, the pollen were mostly dyed blue, and the volumes of pollen grains became larger and regularly spherical. At the terminal flowering stage, about half of the pollen were dyed blue, showing regular spherical shape, and another half were brown, showing irregular empty and shrunken flat shape. The pollen vigor of all the 7 tested *Coix cv* was the highest at the peak flowering stage.

Du et al (2011) believed that addition of boric acid to the

![Figure 4 Induction and Differentiation of Calluses and Embryoids from Anther Culture of Coix CV GDYY.](image-url)
medium could improve the germination rate of pollen. In the present study of nutrient solutions with different concentrations of sucrose and boron acid, it was concluded that the optimal culture medium for pollen in vitro culture of different Coix cv was somewhat different; the optimum liquid medium culture for pollen culture of GDYY, GXY and HBYY was 15% sucrose + 0.01% boric acid, and for the rest cv, 10% sucrose + 0.01% boric acid. And the optimal temperature and duration were 25 °C and 5~7 h respectively for all the tested Coix cv.

As observed, there were considerable disparities between the pollen vigor and the germination rate. The pollen vigor of the 7 Coix cv at peak flowering stage was about 85~95% as estimated by I2-KI staining, while the germination rate determined by liquid medium culture was only about 50%. Similar results were reported by Wang et al (2000) and Li et al (2009) respectively on Phyllostachys praecox and Phyllostachys nigra respectively. The reason may be the resolution of I2-KI staining, which can stain not only the mature pollen with more starch but also some immature and even aborting pollen, and thus make the vigor estimates somewhat higher than the pollen germinating rate, which was based on the growth of pollen tubes in liquid culture.

The pollen grains at the early flowering stage of Coix showed that the uni-, bi- and tri-nucleate microspores coexist in different ratios. Lu et al (2005) reported that microspores at the late uninucleate phase is more desirable for anther culture and better induction. The microspores at the early flowering stage of Coix were spherical with small vacuoles. The male panicle was 1.8~2.1 cm in length, the inflorescence was compact, and the longitudinal diameter of flower bud was 0.8~1.0 cm. The anthers were light yellow, 0.4~0.5 cm in length, and the stigma was fresh and tender, 1.2~1.6 cm in length, and the caryopsis was dark green. The anthers of Coix with the above features at this early flowering stage are suitable for use in anther culture.

The coexistence of calluses and embryos in the same medium were observed during Coix anther culture. Yang et al (1979) on cabbage also observed that different anther culture inducers (calluses, embryos, etc) coexist; and generally the occurrence is attributed to different hormone levels. It was suggested that higher levels of auxin in medium induce more calluses. By combining the cytological observation of the origin of callus from anther with callus forming process, Chen and Zhang (2011) believed that the yellow-brown dense calluses at the fracture of filaments at about 25 days were diploid calluses induced from anther wall and filaments, and embryosoids were readily induced when auxin was low or KT was high. However our results showed that calluses and embryosoids from Coix anther culture were simultaneously induced in the same culture medium. It seemed there was no clear relationship between the formation of the various inducers and the hormone status in the medium. Further researches are needed to clarify.

Although the callus induction rates from anther culture were in significant difference among different Coix cv, calluses could be successfully induced from all the 7 cv in the medium of MS+IAA 0.5 mg/L+KT 2 mg/L.

No plantlets were induced from calluses and embryos from Coix anther culture. Part of the reason might be the recalcitrance of the chimeric calluses induced from Coix anther culture, as reported by Deng et al (2014) on Jasminum sambac and/or the difference in genomic DNA methylation level of the regenerated calluses or embryos as reported by Yao et al (2009) on pakchoi. Or proper inducing and differentiating culture conditions are yet to be identified.

This study for the first time observed the microscopic pollen morphology, compared the pollen vigor, identified the optimal in vitro pollen germination conditions, and established the anther culture and callus proliferation system of Coix cv and laid an excellent basis for induction and breeding of pollen haploid seedlings of this understudied and underutilized food and medicine homologous crop.

Acknowledgments

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Conflict of interest

The authors declare no conflict of interest. We have no financial and personal relationships with other people or organizations that can inappropriately influence our work. There is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the manuscript entitled.

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