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EFFECT OF DOSE FERTILIZER AND CULTIVARS TO THE ACTIVE COMPOUND GLYCERYL TRIOLEATE OF *Coix lacryma-jobi* L.

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ABSTRACT

In order to provide a method for quality control of glyceryl trioleate in *Coix lacryma-jobi* L., this paper focused on differentiating the glyceryl trioleate compound in 14 cultivars and effect of nitrogen on glyceryl trioleate in *Coix* seed by clustering of High Performance Liquid Chromatography (HPLC). This research was carried out to find a variety that has active glyceryl trioleate with the highest content and to investigate the effect of nitrogen to the active ingredients of glyceryl trioleate in *Coix lacryma-jobi* L. and its effect in *Coix lacryma-jobi* L. treatment. The standard regressive curve equation of glyceryl trioleate obtained by using HPLC was: $Y = (7.10^6) X- 495293$, $R^2 = 0.9997$. The average reproducible experiment result of glyceryl trioleate is 0.097, RSD (relative standard deviation) is 1.95%; recovery rate is 97.69% and RSD is 0.89%. The experiment measured the glyceryl trioleate content of 14 varieties of *Coix lacryma-jobi* L using Qianyin #1 (C14). The result showed that fertilizer combination N10F2 (4:1:2:3) produced the highest glyceryl trioleate content in *Coix lacryma-jobi* L. Thus, nitrogen that applied in the fertilizer for 150kg ha^{-1} and the ratio of pre plant application including seedbed application, tillering application and earing application were optimized at 4:1:2:3 ratio.

Keywords: *Coix lacryma-jobi* L.; ELSD parameter; glyceryl trioleate; High Performance Liquid Chromatography (HPLC)

INTRODUCTION

Coix lacryma-jobi L. is a member of the Poaceae family. It is very popular and widely used as a traditional medicine around the world, especially in China. *Coix* root has been used to treat urinary tract infection and inflammation, calculus of kidney, hyronephrosis, rheumatism bone pain, children diarrhea, transparency tropical, menstrual disorders and could eliminate roundworm and worms (Hsu, Lin B. F., Lin J. Y., Kuo, & Chiang, 2003; Ukita & Tanimura, 1961). *Coix* seed is a tonic, able to give a good results either to adults or children. It is used as a cure for milk supply for breast-feeding women (Lee, Lin, Cheng, Chiang, & Kuo, 2008; Lợi, 2006). Recently, studies that were conducted domestically and internationally showed that *Coix* really helps lowering the level of blood sugar and able to fight against cancer (Huang, Chiang, Yao, & Chiang W., 2005; Kim et al., 2004; Li, Chen, Lin, Chiang, & Shih, 2011; Takahashi, Konno, & Hikino, 1986).

Although it has many advantage values and is being widely used, the production of *Coix* is still unable to meet its demand. *Coix* mainly grows in the wild and is scattering cultivated in small scale in Yunnan, Fujian and some Northwest provinces in China. It is very important to enlarge the area for cultivating *Coix* with GAP (Good Agricultural Practices) and find the high quality and high yield of *Coix* seeds. Wan (2010) proved that application of potassium and phosphate fertilizer able to raise triolein content in *Coix* up to 74.1%, while the application of nitrogen fertilizer had no effect on triolein content. Thus, this research was done with two main objectives such as: 1) to differentiate the compound of glyceryl trioleate in the *Coix* seed among different *Coix* cultivars using HPLC method, and 2) to determine the effect of N on glyceryl trioleate in *Coix* seed using HPLC method.

MATERIALS AND METHODS

Plant Materials

Table 1 below shows fourteen *Coix* cultivars used in this study, in which thirteen *Coix* cultivars were often cultivated in China and one *Coix*

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cultivar often cultivated in Vietnam, named Yuenanhuake mi (C13).

Table 1. Fourteen *Coix* cultivars used in this study

Convention	Cultivars
C1	Guangxi longlin#1
C2	Guangxi longlin#2
C3	Guangxi xilin #1
C4	Pinzhong #0
C5	Pinzhong #1
C6	Pinzhong #2
C7	Pinzhong#10
C8	Pinzhong#11
C9	XYYBT
C10	ZYYB12
C11	XYYH12-1
C12	Xingrenxiaobaike
C13	Yuenanhuake mi
C14	Qianyin #1

Experimental Design

The experiment was conducted in the farm yield of Guangxi University (22°51' N, 108°17' E, 78 m asl), Nanning, Guangxi Province, China in early *Coix*-growing season in 2014. The site was located in a subtropical monsoon climate zone. Daily mean temperature and solar radiation during the *Coix*-growing season were 25.4°C and 12.3 $MJm^{-2}d^{-1}$, respectively (Vantage Pro2 weather station, Davis Instruments Corp., Hayward, CA, USA). The soil in field *Coix* paddy field were taken from the research farm. The soil was an Ultisol (USDA Taxonomy) with pH 6.62, organic matter 30.59 g kg^{-1}, NaOH hydrolysable N 129.5mg kg^{-1}, Olsen P 10.08 mg kg^{-1} and NH_4OAc extracted K 72.94 mg kg^{-1}. Experiments with one experimental factor were used. 14 cultivars, labeled from C1-C14 were set as independent variable. Each treatment was replicated three times, placed in random complex blocks. Area of each plot was set 10 m^2. The total area of experiment was 420 m^2.

The fertilizers used 225kg N + 375 kg P_2O_5 + 300 kg KCl for each hectare. The fertilizers method included: pre plant application 40% N + 100% P_2O_5 + 50% KCl; seedbed application 15% N; tillering application 40% N + 50% KCl; earing application 5% N. Size of cultivating places were specified 55 cm × 40 cm.

In 2015, Qianyin #1 was cultivated at the farm of Guangxi University. Three levels of nitrogen fertilizer were applied: 150 kg ha^{-1} (N10), 225 kg ha^{-1} (N15) and 300 kg ha^{-1} (N20). Three managements were set for each level and the formula of pre plant application were: seedbed application; tillering application; earing application; each formula was adopted and respectively specified as 5: 2: 3: 0 (F1), 4: 1: 2: 3 (F2) and 3: 3: 2.5: 1.5 (F3). Thus, nine experimental treatments were formed, namely, N10F1, N10F2, N10F3, N15F1, N15F2, N15F3, N20F1, N20F2 and N20F3. In addition, a reference treatment (N0) of non-nitrogen fertilizer was set. The experiment was randomly split into 30 subplots with each covering of 30 m^2. Each treatment was replicated three times. Fertilizers: pre plant application (375 kg of superphosphate P_2O_5 ha^{-1}) + (150 kg KCl ha^{-1}); Tillering application, 150 kg KCl ha^{-1}; Row spacing and cultivar spacing were specified as 55cm × 40 cm.

Data Collection

Instrument and Reagent

The experiment was using Waters Acquity UPC2 USA, ELSD, EmPower Color Spectrum Management System, PL203 electronic balance, KQ-5200DE numerical control ultrasonic cleaner, 5μl microliter syringe and 0.45 micro-perforated filter membrane. Acetonitrile and dichloromethane were both chromatographically purified. The glyceryl trioleate reference was provided by Shanghai Yuanye Bio-Technology Co., Ltd, batch number 122-32-7.

Chromatographic Analysis Condition

Hibar©C18 chromatographic column ($UPC^2$2-EP 3.6 mm x 150 mm, 1.7 μm), acetonitrile, monochloromethane and dichloromethane were the mobile phase, flow rate was 0.5 ml min^{-1}, column temperature was 28^0C; ELSD parameters: drift tube temperature 70^0C, spray tube temperature 25^0C, nitrogen gas flow rate was 1.2 L min^{-1}.

Solution Preparation

Preparation of test article solution: took 0.6 g powder (filtered by Sieve 3) through precise weighing, added 50 ml mobile phase through precise weighing, soaked the powder for 2 hours and placed it in ultrasonic treatment for 30 minutes then taken out and cooled and weighed, then compensated the weight loss using mobile phase, filtered with 0.45 micro-perforated filter membrane, and finally took the subsequent filtrate as the finish solution.

Preparation of reference solution: an appropriate amount of glyceryl trioleate reference was taken through precise weighing, added mobile phase to formulate the finish solution up to every 1 ml contains 0.14 mg of glyceryl trioleate reference.

Exploration on Linear Relationship

An appropriate amount of glyceryl trioleate reference (0.14 mg ml^{-1}) was taken through precise weighing and mobile phase was added and diluted at 1, 2, 4, 6, 8 and 10 times, respectively and precisely drew 2 µL for sampling and then the peak area was measured.

Precision Test

The same test article solution was taken through 2 µL and implemented samples repeated for 6 times, then the integral value of peak area were measured to analyze the precision.

Stability Test

The same test article solution was taken and analyzed through sampling at 2, 4, 6, 8, 12 and 24 hours in accordance with above-mentioned chromatographic condition, then the integral value of peak area was measured to check the stability of the test article solution.

Reproducibility Test

Six samples of the same test article were taken through precise weighing, then the article solution test was prepared in accordance as required.

Recovery Rate Test

Six samples of known contents were taken through precise weighing, then a precisely-weighed amount of triolein reference was added, the article solution test was prepared as required by instructions and then the peak area and the contents were measured.

Measurement of Triolein Content (USP-http://hmc.usp.org)

The logarithms of peak responses were plotted versus the logarithms of glyceryl trioleate concentrations in mg mL^{-1} from the standard solutions and the regression line was determined using a least-squares analysis; or, a linear regression equation was established using a least-squares analysis according to the logarithms of the peak responses versus logarithms of glyceryl trioleate concentrations in mg mL^{-1} from the standard solutions.

The concentration, C, in mg mL^{-1}, was determined by regression line of the relevant analyze in the sample solution or linear regression equation. The percentages of glyceryl trioleate in *Coix lacryma-jobi* L. were calculated separately. Seed taken:

$$\text{Result} = C \times \left(\frac{V}{W}\right) \times 100$$

Where:
C= concentration of the relevant analyte in the sample solution as determined above (mg mL^{-1})
V= volume of the sample solution (mL)
W= weight of *Coix lacryma-jobi* L., seed taken to prepare the sample solution (mg)

Statistical Analysis

Statistical data analysis using IRRISTAT5.0 and Excel 2007 software, and then strain and variety fertilizing amount were included as experimental factor to calculate the significant difference of glyceryl trioleate content in *Coix lacryma-jobi* L.

RESULTS AND DISCUSSION

Exploration on Linear Relationship

The standard curve was drawn with glyceryl trioleate reference (µg) as the horizontal axis and triolein peak area as the vertical axis (y), as shown in Figure 1.

The regression equation was calculated with least square method in accordance with the standard curve: $Y=(7\cdot10^6)X- 495293$, $R^2 = 0.9997$.

As shown in the chromatogram (Figure 2), the peak of retention time of the solution *Coix lacryma-jobi* L. test article was 0.5 minutes which was consistent with glyceryl trioleate.

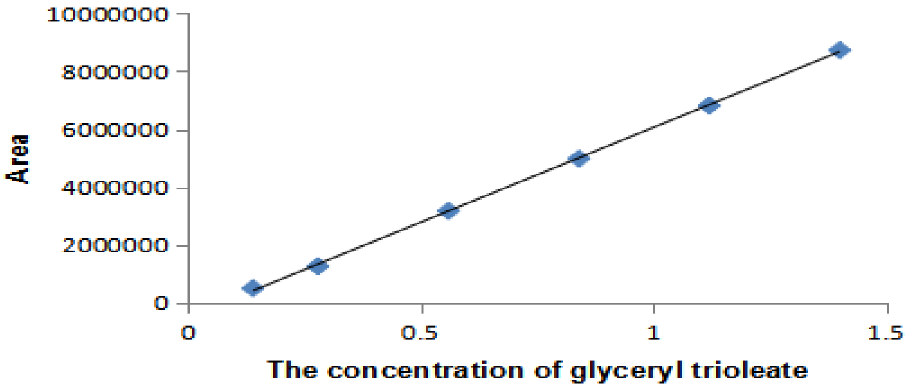
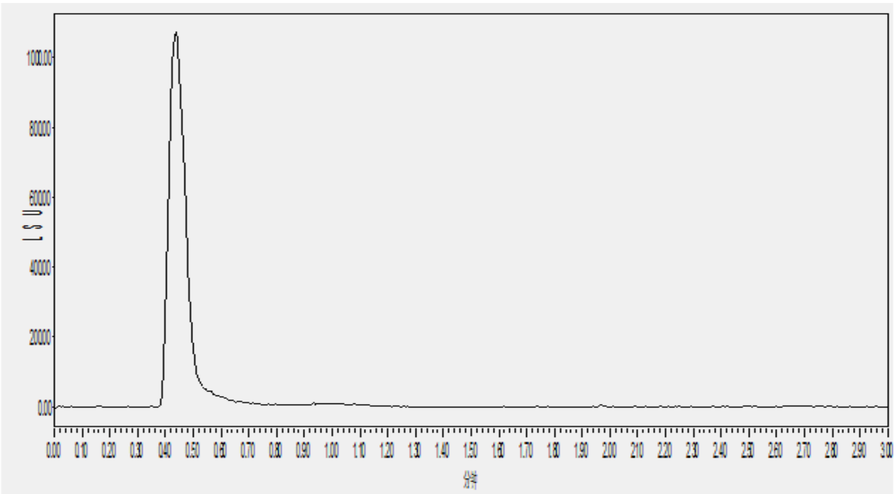
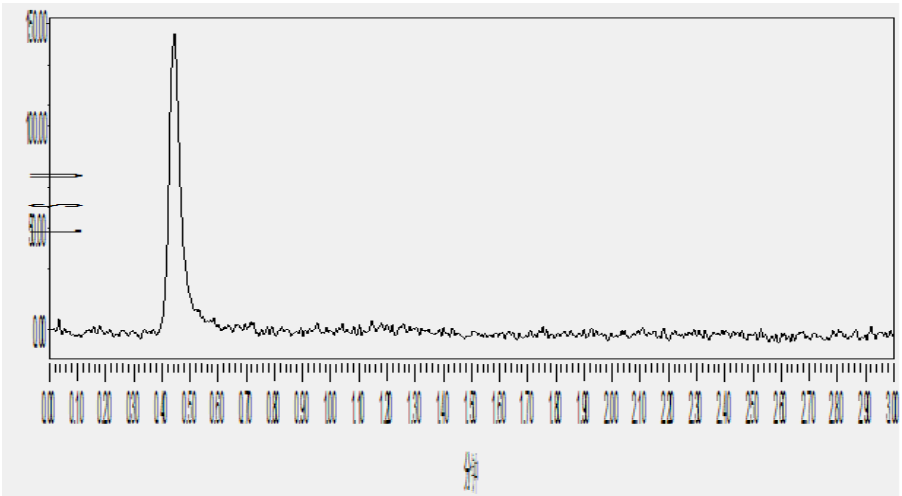


Figure 1. Standard curve for HPLC quantitative analysis of glyceryl trioleate



A



B

Figure 2. HPLC chromatograms of glyceryl trioleate in *Coix lacryma – jobi* L.
(A. Standard of glyceryl trioleate; B. *Coix*)

Precision Test

The measured RSD of glyceryl trioleate peak area was 1.096%, indicated a good precision (Table 2).

Stability Test

The measured RSD of glyceryl trioleate peak area within 24 hours was 0.767, indicated that the test article solution was basically stable within 24 hours (Table 3).

Reproducibility Test

The average content of glyceryl trioleate was measured at 0.097μg, RSD was 1.95% (Table 4).

Recovery Rate Test

The recovery rate of glyceryl trioleate was measured at 95% to 105%, the average recovery rate was 97.69% and RSD was 0.89% (Table 5).

Table 2. Precision of glyceryl trioleate in *Coix lacryma – jobi* L. (n=6)

Repeat times	1	2	3	4	5	6	Average	RSD%
Peak area	240556	245238	230411	226127	240100	229231	235277.1667	1.096

Table 3. Stability of glyceryl trioleate in *Coix lacryma – jobi* L. (n=6)

Repeat times	2h	4h	6h	8h	12h	24h	Average	RSD%
Peak area	276313	270760	283839	285007	275935	279980	278639	0.767

Table 4. Reproducibility of glyceryl trioleate in *Coix lacryma – jobi* L. (n=6)

Repeat times	1	2	3	4	5	6	Average	RSD%
Glyceryl trioleate content（μg）	0.095	0.098	0.100	0.095	0.097	0.097	0.097	1.95

Table 5. The results of recovery test

Repeat times	1	2	3	4	5	6	Average	RSD%
Glyceryl trioleate recovery	97.13	96.18	98.63	98.04	97.98	98.21	97.69	0.89

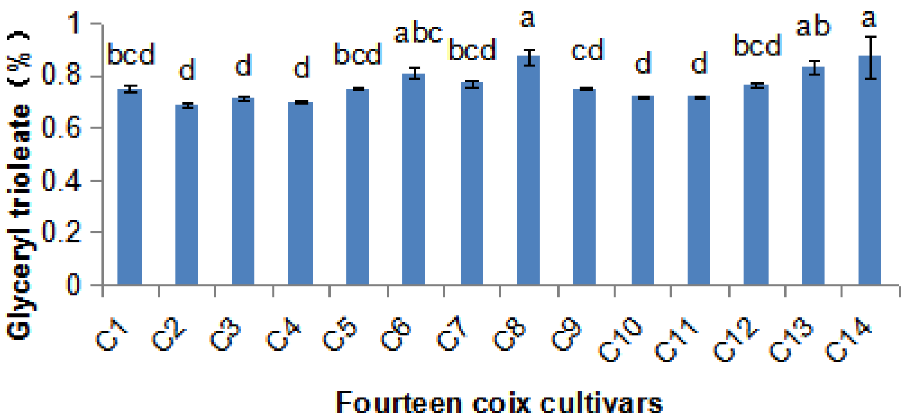


Figure 3. The effect of *Coix* seed on the content of glyceryl trioleate in *Coix lacryma – jobi* L.

Triolein Content Difference between Varieties of *Coix lacryma-jobi* L.

As shown in Figure 3, the glyceryl trioleate content of 14 *Coix lacryma-jobi* L*.* cultivars ranged from 0.69% to 0.87% reached permitted level according to SPCPRC (2015) which stated that any value under 0.5% will not be permitted.

The cultivars was divided into 3 groups by glyceryl trioleate contents and from 14 cultivars, divided into 3 groups: High glyceryl trioleate: C6, C8, C13 and C14; Average glyceryl trioleate: C1, C5, C7, and C12; Low glyceryl trioleate: C2, C3, C4, C9, C10 and C11. The differences of glyceryl trioleate content among the groups were significant. Therefore, the content of the first group was higher than second and third group at 95% confidence level. Specifically, C8 and C14 had the highest glyceryl trioleate content and shared the same characteristics with C13 but another 11 varieties demonstrated significant difference at 95% confidence level.

Impact of Nitrogen Management on the Triolein Content in *Coix lacryma-jobi* L.

As shown in Figure 4, under different nitrogen application level, at 95% confidence level, all the treatments have significant differences in glyceryl trioleate content compared with N0 treatment (without fertilizer application) except for the N20F3 treatment.

The treatment of *Coix lacryma-jobi* L. when compared in the same nitrogen level, but using different in fertilizer methods (F1, F2, F3), at 95% confidence level on 150 kg ha^{-1}, there was no difference between all the fertilizer methods in glyceryl trioleate content. At 225 kg ha^{-1} there was difference between F2 and F3, while at 300 kg ha^{-1}, between F1 and F3 was also have a differences. By combining the nitrogen levels with the fertilizer methods, results showed that no differences between N10F2 and N15F2, and also between N15F3 and N20F3 in glyceryl trioleate content but there were significant differences between N10F2, N15F2 and N15F3, N20F3 at 95% confidence level. Thus, it found that nitrogen levels and the fertilizer methods have significant effect on glyceryl trioleate content in *Coix* seed in this experiment which N10F2 and N15F2 had the highest content and N10F2 (4:1:2:3) was preferred for the fertilization amount that has lowest nitrogen level.

Pharmacology has proved that *Coix lacryma-jobi* L. oil can inhibit tumor cell growth and regulate immunologic functions. The Kanglaite injection (with *Coix lacryma-jobi* L. oil as the active constituent and triolein which is the major constituent of *Coix lacryma-jobi* L. oil), as an intravenous preparation, has been applied clinically and has produced favorable outcomes in the treatment of liver cancer. Takahashi, Konno, & Hikino (1986) isolated 3 substances which are Coixan A, Band C from the *Coix* seed (*Coix lacryma-jobi* L. var. ma-yuen) and showed that *Coix* seed extracts and these 3 substances has hypoglycemic effects in mice (Lu, Wu, Dong, & Li, 2009; Lu, Li, & Dong, 2008; Takahashi, Konno, & Hikino, 1986).

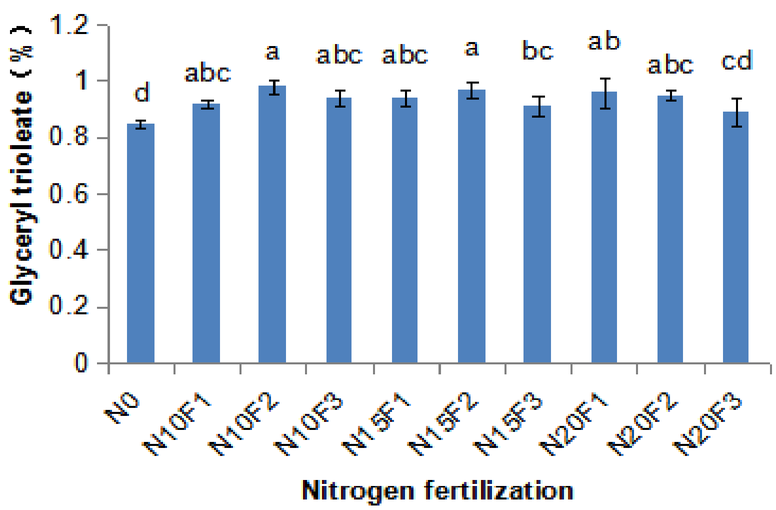


Figure 4. The effect of fertilization on the content of glyceryl trioleate in *Coix lacryma-jobi* L.

Lee, Lin, Cheng, Chiang, & Kuo (2008) studied the effect of *Coix* stem extract and 5 lactam compounds separated from *Coix* stem extract by using Methanol (MeOH) on tumor cell inhibition. The experiments were conducted on A549 cell – human lung cancer cells; H-29 cells – colorectal cancer cell, etc. The results showed that *Coix* stem extract at all plant growth stages and 5 lactam compounds had cancer cell inhibition effect. The extract separated from *Coix* seed powder by using alcoholic was reported that had significant effect on reducing the number of primary follicles precancerous abnormalities (Li, Chen, Lin, Chiang, & Shih, 2011).

Huang, Chung, Kuo, Lin, & Chiang (2009) have investigated the effects of *Coix* seed coat extracts (*Coix lacryma-jobi* L. var. ma-yuen Stapf) on lipopolysaccharide induced inflammatory response in RAW 264.7 macrophages and shows seed coat extract of *Coix* has anti-inflammatory effects against the proliferation of cell proliferation nitric oxide and prostaglandin E2 induced by lipopolysaccharide by reducing the production of nitric dioxide synthase enzymes and cyclooxygenase.

CONCLUSION AND SUGGESTION

In this study, the glyceryl trioleate contents in all 14 varieties of *Coix lacryma-jobi* L. oil were all above 0.69%. Qianyin #1 (C14) and Pinzhong #11 (C8) had the highest glyceryl trioleate content. Since the growth time of Pinzhong #11 (C8) was longer, Qianyin #1 (C14) was chosen for the nitrogen management experiment. The nitrogen management experiment showed that treatment N10F2 (4:1:2:3) had the highest glyceryl trioleate content in *Coix lacryma-jobi* L. oil, thus the implemented nitrogen management at 150 kg ha^{-1}, and the ratio of pre plant application: seedbed application: tillering application: earing application was optimized at 4:1:2:3 ratio.

By optimizing *Coix* seed and its cultivar treatment, it can be targeted for the development to enhance the glyceryl trioleate content as functional in the traditional medicine. The other compounds that contained in *Coix* seed should also further investigated. In addition, their ability in the medical treatment need further studies in the future.

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