LRF-785
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RESEARCH ARTICLE Legume Research- An International Journal

Optimum Seed Harvest Time of *Trifolium lupinaster* L. in Relation to Flowering, Pods and Seed Characteristics

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10.18805/LRF-785

ABSTRACT

Background: Pod splitting is the main cause of the low seed yield of legume forage grasses. In the present study, the flowering pattern, seed maturity characteristics and the optimum seed harvesting time of *Trifolium lupinaster* L. were investigated to improve seed yield and quality.

Methods: All the required observations for this study were completed in the new line nursery from July to October 2022. Flowering habits of populations, individual plants, inflorescences and florets were observed for two consecutive months from July 1 to September 1 and daily temperatures and humidity were recorded.Observations on the state of seed and pod development were made by sampling and measuring in three batches every three days from July 25 to August 23 starting from the 12th day after the first flower and continuing until the first day of the season. Starting from July 15, seed yield and constitutive factors were measured every 10 d until September 5th.

Result: The results showed that the flowering period of *T. lupinaster* L. population lasted for 60 days and the peak opening period was on the 11th-20th day after heading began, the florets opened most vigorously at 11:00-13:00 every day and inflorescences were opened more vigorously at 15:00 every day. The opening pattern of florets and inflorescences was greatly affected by temperature and humidity. The fresh weight of pod skin and seeds reached the maximum on the 15th day after flowering; the length and width of pods and seeds reached the maximum on the 18th day; the water content of pod skin and seeds began to decrease sharply on the 21st day, while the pods began to crack and the surface of the seeds dried and hardened; the water content of seeds decreased to below the safe storage moisture at 27 days. Our resuts showed that mid-August was the optimum seed harvesting period for *T. lupinaster* L.

Key words: Flower dynamics, Optimum seed harvest time, Pods characteristics, Seed characteristics, *Trifolium lupinaster* L.

INTRODUCTION

Trifolium lupinaster L. is a perennial legume grass mainly distributed in Inner Mongolia and northeast China. It is a strong cold-tolerant, drought-tolerant and widely adaptable plant. *T. lupinaster* has high medicinal and ornamental value, which is regarded as traditional Chinese medicine. However, the flowering characteristics and the seed maturation characteristics are still unclear, which is very important for determining the optimum seed harvest time of *T. lupinaster*. The low seed yield and poor seed quality may be due to unsuitable seed harvest time (Wang *et al*., 2008). Therefore, lots of research has focused on how to determine the optimum seed harvest time (Wang *et al*., 2008; Jaradat and Rinke, 2008; Wang and Zhou, 2006).

The optimum seed harvest time is influenced by many factors, *e.g*. field management, flowering dynamics (Jaradat and Rinke, 2008), pod shattering, pods and seeds color change, maturity stage, seed moisture content and so on. The seed yield of *Elymus sibiricus* reaches its maximum in 26 days to 27 days after the full bloom, which is the appropriate seed harvest time for *E. sibiricus* (Mao *et al*., 2003). Wang *et al*. (2008) found that the optimum seed harvest time of *Vicia cracca* was when the pods turned light brown and the seeds turned black.

The number of florets per inflorescence, the number of pods per plant and the seed setting rate are the dominant seed yield components(Biabani *et al*., 2021), which are also the key factors to determine the optimum seed harvest time. In the previous studies, the number of pods per plant was considered the most determinant yield component in *Brassica napus* and *B. juncea*, which is highly correlated with the seed yield (Biabani *et al*., 2021). In oilseed crops, the number of seeds is the dominant yield component (Jaradat and Rinke, 2008).

A new line of *T. lupinaster* was formed by natural combined with artificial single-plant hybrid selection method

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How to cite this article: Peng, X., Wu, F., Fu, N., Shi, F. and Zhang, Y. (2024). Optimum Seed Harvest Time of *Trifolium lupinaster* L. in Relation to Flowering, Pods and Seed Characteristics. Legume Research. DOI: 10.18805/LRF-785.

Submitted: 05-12-2023 **Accepted:** 03-01-2024 **Online**: 22-01-2024

in 2012-2016, which has a long blooming period, colorful flowers and upright shape and is targeted to be bred into a new ornamental grass variety. However, due to the characteristics of split pods and grain drop of this line and the best harvesting period of its seeds is not clear, it can not be well promoted and utilized at present. Meanwhile, we found that *T. lupinaster* has stronger resistance compared with *Trifolium pratense* and *Trifolium repens* of the same genus in Inner Mongolia, which can be a high-quality genetic resource for *Trifolium* forage grass to enhance its resistance. In order to lay the foundation for the next step of distant hybridization, it is important to investigate the flowering habit of *T. lupinaster*. Therefore in this study, our objectives were to (1) model the flowering dynamics, (2) investigate the pods and seed development characteristics and (3) determine the optimum seed harvesting time of *T. lupinaster*. Our study will provide a theoretical basis and guidance for the seed production of *T. lupinaster*.

MATERIALS AND METHODS

Materials and experimental location

In 2011, our research team collected wild *T. lupinaster* seeds in the Daqingshan area of Inner Mongolia (40°59′25.82″N, 111°58′52.33″E, Inner Mongolia) and raised seedlings in a greenhouse in the same year. 2012 saw the transplanting of seedlings cultivated in the previous year in the pasture nursery of Inner Mongolia Agricultural University (40°482 24.82″N, 111°43′30.76″E), successfully establishing a seedling nursery for wild fireball breeding resources. 482 24.82″N, 111°43′30.76″E) pasture nursery, transplanted seedlings cultivated in the previous year and successfully established a *T. lupinaster* breeding resource nursery, which consisted of three replicated plots of 25 square meters (5 m × 5 m) each and the soil type of the plots was sandy loam. The area has a temperate semi-arid continental monsoon climate, with an altitude of 1050 m above sea level, an average annual precipitation of about 350-400 mm and an average annual temperature of 6.7°C. A new strain of *T. lupinaster.* was formed by the natural combined with artificial monoculture hybrid selection method in 2012-2016, which has a long blooming period, colorful flowers and an upright shape and it is expected to be a new variety of ornamental grass. The relevant experiments involved in this study were conducted from July to October 2022, during which the experiment was completed in a natural environment.

The dynamics of flowering

100 inflorescences with existing buds were randomly selected for observation. The number of inflorescences and the daily average temperature and humidity were recorded until the flower bloomed. The percentage of the flowered inflorescences was recorded.

10 inflorescences that were about to open were selected randomly. The number of open florets of each inflorescence was counted each hour between 8:00 to 18:00 during 3 consecutive days. The stamens of the open inflorescences were removed by scissors and the temperature and relative humidity were recorded. The average number of flowering florets at a different time in the three days were counted.

10 florets that were about to open were selected randomly. The flowering status was observed each hour from 8:00 to 18:00. The open florets were recorded as 1 and the unopened florets were recorded as 0. Then, the peak of the flowering florets number was induced by the average.

The characteristics of the seeds and pods

The healthy seeds were selected and planted in the plots (5 m × 5 m) in single rows and plants, the distance between the plants was 50 cm. The plots were repeated 3 times, the other conditions were the same.

70 inflorescences which about to open were marked randomly in the bud stage and the sample would be collected every 3 days on the 12th day marked and end on the 30th day. The samples were collected three times as Table 1. 10 pods were collected from the 70 marked inflorescences for the observation of the pods and seeds.

The pods will be observed and photoed by the microscope and the picture will be analyzed by the Digitizer 4.2 software to get the length (mm) and width (mm). The thickness (mm) of the pods was measured by the Vernier caliper.

The fresh weight of the pod shell and seeds was measured by the electronic balance. Then, the pod shell and seeds were dried at 80°C to a constant weight and weighed to an accuracy of 0.0001 g. The water content was calculated as the formula:

$$\text{Water content (\%)} = \frac{\text{Fresh weight - Dried weight}}{\text{Fresh weight}} \times 100$$

Seed yield and its components

Three quadrats were selected randomly avoiding marginal effects every 10 days from July 15 to September 5 (2022). The seed yield of a single plant (g/m^2) and the seed yield above ground (g/m^2) were investigated. Three single plants were selected randomly and 5 inflorescences were selected in every plant randomly. The florets number per inflorescence, the pods number per inflorescence, the split pods per inflorescence, the seeds number per pod and the thousand seed weights (g) were counted. Then, the pod splitting rate (%) and the podding rate (%) were calculated

Table 1: The sample collection time.

	Collection time (Month/day)						
The first group	07/25、	007/28、	07/31、	08/03、	08/06、	08/09、	08/12
The second group	08/01、	08/04、	08/07、	08/10、	08/13、	08/16、	08/19
The third group	08/08、	08/11、	08/14、	08/17、	08/20、	08/23	

and finally, the actual seed yield per period was converted (g/m^2).

Statistical analysis

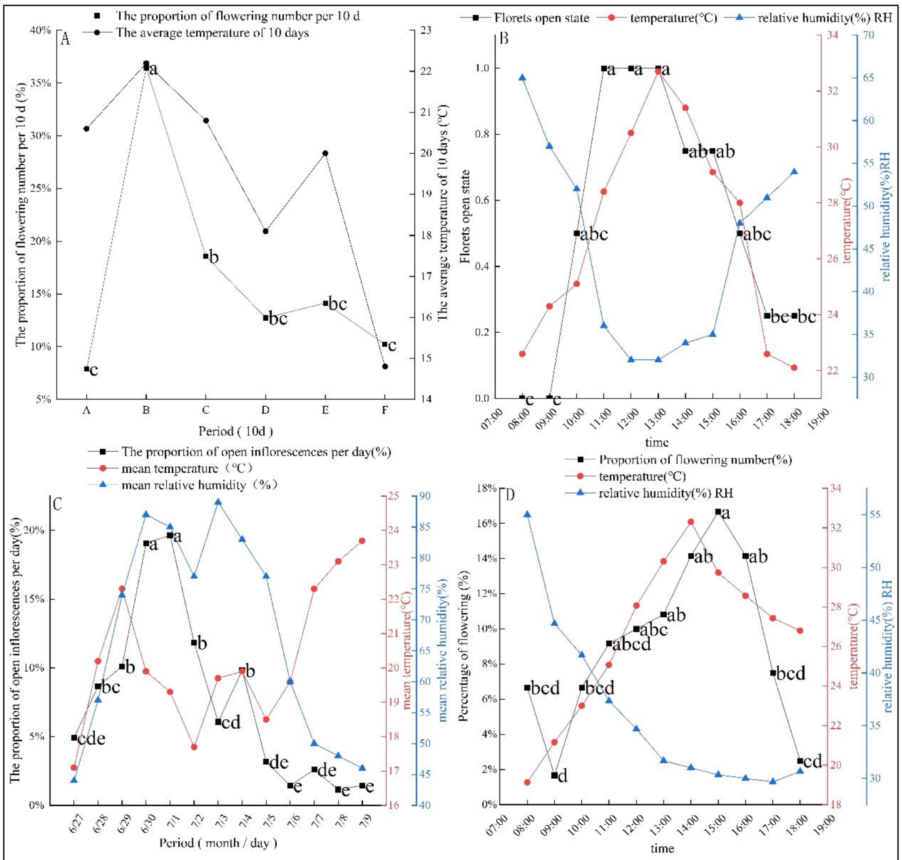
One-way ANOVA was performed to compare differences in pods in terms of fresh weight, dry weight, pod splitting rate, water content, *etc*. The analysis was calculated by SAS 9.0 software and plotted by Excel 2019 software.

RESULTS AND DISCUSSION

The dynamics of flowering

Flowers, as the reproductive organs of angiosperms, have been one of the research hotspots of plant reproductive ecology (Xiao *et al*., 2004). In addition to the genetic characteristics and physiological conditions, the flowering characteristics and dynamics of plants are also affected by the environmental abiotic flowers (*e.g*. temperature, humidity, *etc*.), which may influence the flowering period or the daily flowering time (Shi *et al*., 1996; Wu and Du, 2000). Furthermore, studying the flowering habits of plants is helpful to the breeding of improved varieties. The characteristics of plant flowers include single-flower characteristics and group-flower characteristics. Single-flower characteristics generally refer to the color, size, odor, *etc*., while group-flower characteristics refer to the spatial arrangement of inflorescences and the dynamics of flowering. The inflorescences of *T. lupinaster* L. are bright in color and open in a concentrated period, which is conducive to insect pollination. The observation period in the study was from July 1 to September 1, the opening period of the population could last more than 60 days (Fig 1A) and the highest percentage of flowering number (36.46%) was recorded on the 11th-20th day flowering number gradually decreased with the extension of the opening period.

The inflorescence is a capitulum and there are 10-40 florets in each inflorescence. The peak anthesis of inflorescences is 4 d to 5 days after heading began and most inflorescences bloom from 14:00 to 16:00. Inflorescence opened most vigorously at 15:00 when the temperature was 29.7°C and the relative humidity was 30%. The opening of inflorescence in one day was consistent with the temperature change and the response to temperature changes shows hysteresis. It had no obvious relationship with the change in relative humidity. The inflorescences



A graph represents the opening duration of the population. In the abscissa, A represents 0-10 days after flowering, B represents 11-20 days, C represents 21-30 days and D, E and F are successively extended for 10 days. B-chart represents the dynamics of floret opening day; the C diagram represents the duration of inflorescence opening ; the D diagram represents the daily dynamics of inflorescence opening.

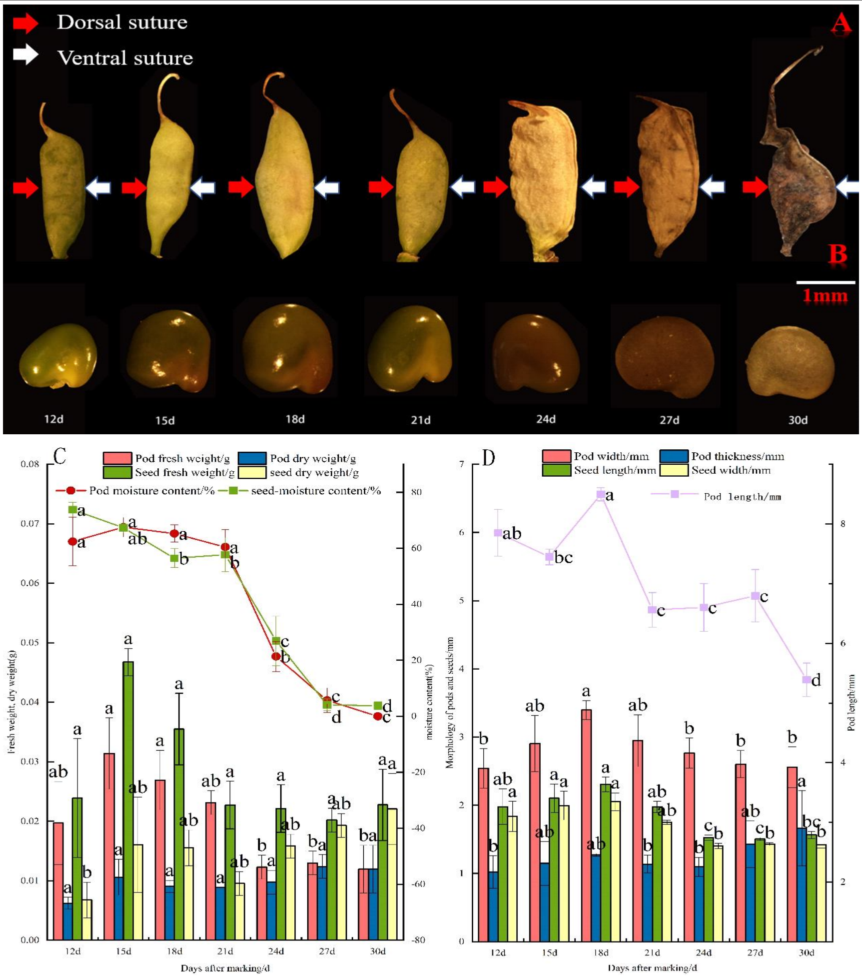
Fig 1: Daily dynamics of florets and inflorescences and duration of population and inflorescence opening.

opening lasted for 13 days (Fig 1C), reached its peak on the 5th day after opening and then declined gradually. The opening of inflorescence was greatly affected by temperature and humidity and although it was consistent with the change of temperature and humidity, however, the response to temperature changes shows hysteresis. For example, the temperature had risen on July 3, but the proportion of inflorescence opening number did not rise accordingly, but was still decreasing due to the cooling effect on July 2 and showed an upward trend on July 4.

The flower opening state is high from 10:00 to 16:00 in a day and the peak period is from 11:00 am to 13:00 (Fig 1B). The appropriate temperature and the relative humidity for florets to open is 25.1°C-31.4°C and 34%-52%, respectively. The daily opening dynamic of the floret is consistent with the temperature change basically, but contrary to the change in relative humidity. The open time of each floret could last 5 h to 8 h and most of the florets bloom from 11:00 to 13:00. The changing pattern of the percentage of flowering was consistent with the changing pattern of the percentage of flowering was consistent with the changing pattern of the average temperature in each period and the average temperature only increased by 1.9°C from D to E, while the percentage of flowering increased by 1.39% at the same time. The flowering time and number are affected by the weather conditions. The flowering habits of *T. lupinaster* mentioned above have not yet been reported in the previous studies, which are similar to the flowering habits of Trifolium repens. The florets flowering time of Trifolium repens is mainly concentrated from 12:00 to 16:00 and last for 2 h to 4 h (Wang and Zhang 2016).

The characteristics of pod and seed

The morphology changing tendency of pods and seeds with the sampling time was shown in Fig 2A and 2B. On the 21st day after heading began, the pods began to split, the pod shell skin is dry pleated and dark in color and mold appeared on some pods. From this point on, the seed coat gradually



(A) indicates phenotypic changes during 12-30 d pod development. (B) Indicates changes in appearance during 12-30 d seed development. (C) Trends in seed and pod skin fresh weight, dry weight and water content from 12-30 d. (D) Trends in seed and pod volume indices from 12-30 d.

Fig 2: Development dynamics of pods and seeds.

loses its luster, darkens in color, dries out and hardens. The water content of the pod shell and seeds decreased continuously with the sampling time (Fig 2C) and reached 73.72% and 73.82% on the 12th day respectively. However, the water content of the pod shell and seed decreased to 73.82% and 67.65% respectively, while the fresh weight was highest on the 15th day. The water content of pod skins and seeds began to decrease significantly on the 21st day and fell below the safe storage water value. The length and width of the pod and seed increased firstly and decreased with the sampling time and reached the maximum value on the 18th day (Fig 2D). The suitable seed harvest time of different species can be deduced by the developmental dynamics of pods and seeds, which are based on the number of days after pod or caryopsis bloom (Wang, 2008). The seed yield of Elymus sibiricus reaches its maximum in 26 days to 27 days after the dates of peak anthesis, which is the appropriate seed harvest time for *E. sibiricus* (Mao *et al*. 2003). In the present study, the seeds become dry and hard and the moisture content of *T. lupinaster* fell below the safe storage moisture content on the 27th day after heading began, which is the suitable seed harvest time for *T. lupinaster*. The morphological indicators of pods or seeds (color, size, *etc*.) are also considered to be the simple, fast and accurate indicators to determine the suitable seed harvest time (Wang, 2008; Lin *et al*., 2013; Dong, 2007). The color of pods or seeds could change regularly with the degree of maturity during the growth and development of most plants. Morphological indicators observation could save a lot of time needed for seed physiological indicators and quality measurements. Wang *et al*. (2008) found that the optimum seed harvest time of *Vicia cracca* was when the pods turned light brown and the seeds turned black. In the present study, the width and length of the pods and seeds increased first and then decreased with the increase of development time after flowering. The length and width of pods and seeds reached the maximum on the 18th day after flowering, while the water content was relatively high. The water content of pod shells and seeds began to decrease significantly on the 21st day after heading began and fell to below the safe storage moisture value on the 27th day. The color of pods and seeds changed from bright green to brownish-green during the 12th to 21st day and turned to yellowish-green on the 21st day. The seed color turned light brown on the 27th day and the pods turned brownish black. The result of the present study showed that the yield and quality of seeds were optimal on the 27th day, which was exactly in the middle of August, which is the optimal seed harvest time of *T. lupinaster*. Our result showed that the optimum seed harvest time of *T. lupinaster* could be deduced by the development dynamics and color changes of pods and seeds. However, the above methods do not apply to all plants. There were no visible changes in seed color during the seed development of Hordeum brevisubulatum, so the appropriate seed harvest time could not be inferred by the indicator (Wang *et al*., 2006).

In conclusion, the dynamic changes of pod and seed development showed that it was ideal to harvest seeds on the 27th day after flowering, the water content of the seeds reached safe water content in that time. If the seeds were harvested before 27d, the water content was too high to store. The water content of seeds was too high to store if we harvested seeds before 27d, while the high seed drop rate and severe mildew resulted in unsafe seed storage if the seeds were harvested after 27d.

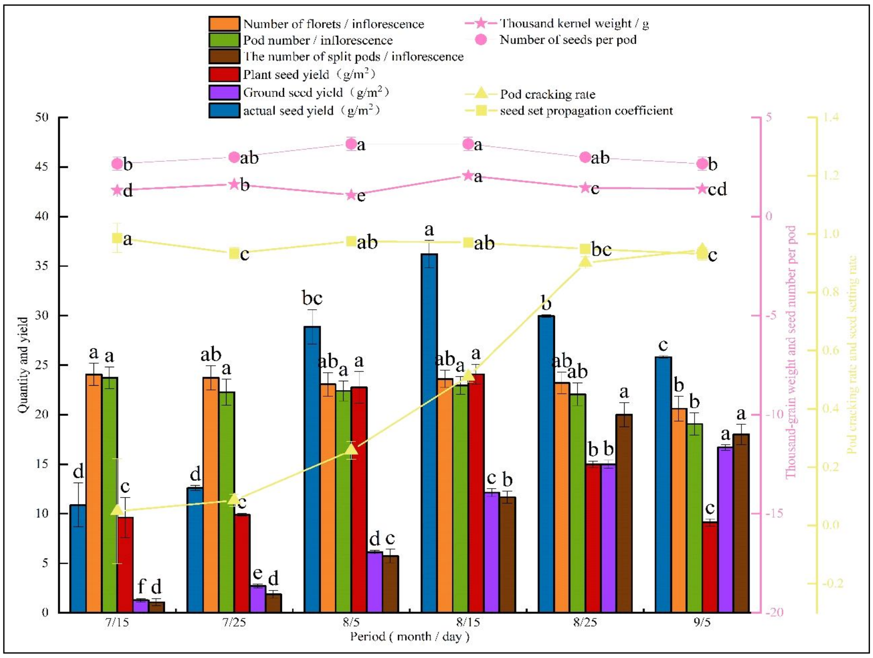


Fig 3: Analysis of seed yield and its components in different periods.

Seed yield and its component

The number of flowers per inflorescence, the pod number, the seeds number per pod and the seed setting rate were less affected by sampling time. The pod splitting number per inflorescence increased gradually with the maturity of seeds, which lead to the seed yield above ground increasing continuously. However, the pod splitting rate was very high and the weight of 1000 seeds changed, which led to the plant and actual seed yield first increasing and then decreasing. The highest actual seed yield was on August 15 (8/15), which is because the weight of 1000 seeds is the highest at this time. After this, the weight of 1000 seeds and the seeds per pod declined, which lead to the decrease of the plant seed yield and the actual seed yield (Fig 3). As a result, the best harvest time for seeds is August 15. It is of great significance for the research of forage seed production to study the composition factors of seed yield (Smalle and Jomphe, 1989). It has been shown that seed weight is negatively correlated with days to flowering, fruit length and number of seeds per fruit (Ha *et al*., 2023). Therefore, thousand-seed weight becomes one of the important factors to consider when judging seed yield. The setting percentage, number of inflorescences, thousand seed weight, number of pods and number of florets have been identified as the component factors of forage seed yield. It was found that thousand seed weight increased with increase in moisture content. by studying pigeonpea (Pusa Arhar 16) and lentil (Pusa 4717) (Gupta *et al*., 2023), therefore, when judging the optimum harvesting period, there is a need to focus on thousand seed weight to avoid early harvesting due to high moisture content, which can result in Seed yield loss due to high moisture content. Which will change under different periods of seed maturation and different climatic conditions, resulting in differences in seed yield (Hebblethwait *et al*., 1980; Langer 1980). In the present study, the seed yield was different in different development periods, which was mainly caused by the falling flowers and splitting pods and greatly affected by the thousand seed weight and number of seeds per pod. A study on seed yield components of peanut revealed that pods per plant exerted positive direct effect on seed yield. Thus to improve seed yield, appropriate harvesting period can be selected in the future through artificial interventions in order to increase podding rate and reduce grain fall (Giradhari Lal Yadav *et al*., 2023). The optimum seed harvest time of *T. lupinaster* was on August 15th, which was inferred through the comprehensive analysis of seed yield and yield component factors.

CONCLUSION

The flowering period of the *T. lupinaster* population can last 60 days, the date of the peak anthesis was on the 11-20th day after heading began. The flowering period of a single inflorescence can last 13 days, the full-bloom stage was on the 4-5th day and the daily blooming time of the inflorescences is 15:00. Meanwhile, the daily blooming time of the single floret is from 11:00 to 13:00. The flowering pattern of florets or inflorescences was consistent with the change of temperature and was greatly affected by temperature and humidity.

On the 21st day after heading began, the pods began to split, the seeds turned tarnished, dry and hard and the water content of the pods and seeds began to decline significantly and fall to below the safe storage moisture on the 27th day, which is the best seed harvest time.

The results of the yield composition factor analysis also showed that the best seed harvest time was on the 27th day, which was August 15. *T. lupinaster* has strong sexual reproduction ability and seed production potential, pod splitting is the main reason for the low seed yield.

Synthesizing the three parts of the study on flowering habit, seed and pod development dynamics and seed yield and its components. The results of the three parts of the study were correlated and validated with each other in time. Therefore, we concluded that the best time to harvest seeds of *T. lupinaster* is around August 15 of each year. In conclusion, the results of this study have laid the foundation for seed production and utilization and hybrid breeding of *T. lupinaster*.

ACKNOWLEDGEMENT

The research has been reported by “Inner Mongolia Autonomous Region Seed Industry Science and Technology Innovation Major Demonstration Project” open competition mechanism to select the best candidates’ projects of China (2022JBGS0040).

Conflict of interest

The article is original and has not been published previously, is not under consideration for publication elsewhere and if accepted, it will not be published elsewhere in the same form, in English or any other language. The submission of the article has the approval of the all the authors and the authorities of the host institute where work had been carried out. All the authors have made substantive and intellectual contributions to the article and assume full responsibility for all opinions, conclusion and statements expressed in the articles.

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