# The correlation between the developmental stages of pea microspores and the morphology of flower buds

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**Abstract:** To investigate the correlation between microspore development stage and the appearance and morphology of flower organs in Pisum sativum, the novel pea materials YP58 and YW90 were employed as experimental subjects. Flower buds exhibiting varying diametral ranges were chosen for cytological examination, and the saffron solid green staining method was employed to discern the diverse developmental stages and morphological attributes of microspores. Furthermore, the association between flower bud size and microspore development stage was examined. The findings revealed that the microspore development in both pea materials predominantly encompassed the microspore mother cell stage, tetrad stage, early and middle mononuclear stage and late mononuclear stage, each displaying distinct features. Notably, the development duration of microspores exhibited a close correlation with the longitudinal diameter of the flower bud. The correlation between microspore development progression and the longitudinal diameter of the flower bud was consistent in both pea materials. Therefore, the longitudinal diameter of the flower bud can serve as a primary indicator for assessing the microspore development stage.

## 1 Introduction

Pea (*Pisum sativum* L.), a member of the legume family and the pea genus, is an annual climbing herb also referred to as wheat pea, field pea, or dry peas. In the context of China, pea breeding has been relatively delayed and has progressed at a sluggish pace. The primary methods employed in pea breeding have involved systematic selection, single (double) crosses, and introductions, with the technical approaches lagging behind those of staple crops such as corn and wheat. Conventional breeding necessitates extensive human and material resources for the selection and cultivation of new varieties via sexual hybridization to attain a high degree of pure self-incompatible lines. This process typically entails 5 to 6 years of consecutive generations of artificial self-crossing and selection[1]. The establishment of pure diploids through the duplication of haploids represents a fundamental mode of developing self-compatible lines. Consequently, haploid breeding offers a means to circumvent hybrid segregation, reduce breeding periods, enhance the efficiency of obtaining pure materials, and facilitate the selection and utilization of mutants, thereby serving as a rapid and effective pathway in contemporary plant breeding.

Anther culture represents a highly viable and efficient approach for expeditiously generating homozygous dihaploid plants, and it stands as a principal method within plant haploid breeding [2]. Given that haploid plants possess only one set of chromosomes, recessive variations can be expressed, and subsequent doubling results in the formation of genetically stable homozygous diploids, devoid of character segregation. This feature not only serves to abbreviate the breeding cycle but also holds significant importance in facilitating the study of species and functional genomes within plant cultivation.

In the initial investigations into inducing haploids via anther culture, it was established that the developmental timeline of flower buds directly governs the maturation period of microspores enclosed within anthers, with even minor disparities in microspore developmental timing exerting pronounced effects on haploid induction [3]. Distinct developmental stages of microspores are closely linked to callus formation and subsequent plant regeneration. Consequently, an imperative precondition for obtaining haploid plants through anther culture is the meticulous examination of the cytological development process of microspores [4]. Recognizing the optimal developmental phase of microspores stands as an essential component of anther culture, given its pivotal role in influencing callus formation and plant regeneration. To ascertain the correlation between the developmental stage of pea microspores and the morphological characteristics of flower buds, this study scrutinized the flower bud morphology of novel pea materials YP58 and YW90, alongside the cytological attributes of microspores at varying developmental stages. The significance of this investigation lies in its potential to enable the determination of microspore developmental timing based on flower organ morphology, thereby obviating

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the need for microscopic examination during sample collection. This streamlined approach not only enhances efficiency by saving time and simplifying the material collection process, but also contributes to augmenting the overall effectiveness of anther culture.

## 2 Materials and methods

### 2.1 Materials

YP58 and YW90 pea materials were selected as experimental materials. These two materials are new pea materials selected and bred by Nanchong Academy of Agricultural Sciences.

### 2.2 Methods

#### *2.2.1 Morphological Observation and Measurement of Flower Buds*

Take samples from 9:00 am to 11:00 AM on sunny days. Put buds from healthy plants into sampling bags and refrigerate them for later use. Flower buds with longitudinal diameter of 2-10mm were selected and graded with a difference of 1mm. 5 buds were selected for each level and their longitudinal and transverse diameters were accurately measured.

#### *2.2.3 Cytological observation during the development of microspores*

##### *2.2.3.1 Sampling*

Place the flower buds in 4mL centrifuge tubes and fix them with FAA fixative for more than 24h. Remove the tissues from the fixative in a fume hood and use a scalpel to trim and flatten the tissues of the target site, and place the trimmed tissues and corresponding labels in a dehydrated box.

##### *2.2.3.2 Dehydration and wax immersion*

Put the dehydrating box into the dehydrator for dehydration by sequential gradient alcohol. 75% alcohol 4h, 85% alcohol 2h, 90% alcohol 2h, 95% alcohol 1h, anhydrous ethanol I for 30min, anhydrous ethanol II for 30min, alcoholic benzene for 5-10min, xylenol I for 5-10min, xylenol II for 5-10min, melt paraffin wax at 65℃, melt paraffin I for 1h, melt paraffin II at 65℃, melt paraffin I for 1h, and then melt paraffin I for 1h, then melt paraffin I for 1h, then melt paraffin II at 65℃. I 1h, 65℃ melting paraffin II 1h, 65℃ melting paraffin III 1h.

##### *2.2.3.3 Embedding*

The wax-impregnated tissues are embedded in an embedding machine. First, put the melted wax into the embedding frame, and before the wax solidifies, take the tissues out of the dehydrating box and put them into the embedding frame according to the embedding surface and label them accordingly. Cool at -20℃ on the freezer table. After the wax has solidified, remove the wax block from the embedding frame and trim the wax block.

##### *2.2.3.4 Sections*

The trimmed wax blocks should be cooled at -20℃ on the freezer table, and then the cooled blocks should be sliced in a paraffin slicer to a thickness of 4 um. The slices should be floated on a spreader at 40℃ in warm water to flatten the tissues, and the slides should be fished out, and then slices should be baked in an oven at 60℃. The water should be dried and the wax should be baked and removed. After the water is dried and the wax is baked, take out and store at room temperature.

##### *2.2.3.5 Paraffin section dewaxing to water*

Sequentially put the section into xylene I 20min, xylene II 20min, anhydrous ethanol I 5min, anhydrous ethanol II 5min, 75% alcohol for 5min, tap water washing.

##### *2.2.3.6 Saffron staining*

The slices are stained in Saffron dye solution for 1-2h, and then washed with tap water to remove the excess dye.

##### *2.2.3.7 Decolorization*

The slices are sequentially put into 50%, 70%, 80% gradient alcohol for 3-8s each.

##### *2.2.3.8 Solid green staining*

The slices are put into solid green dyeing solution for 30-60s, and anhydrous ethanol is dehydrated in three cylinders.

##### *2.2.3.9Transparent sealing*

The slices are transparent in clean xylene for 5min, and the slices are sealed with neutral gum.

##### *2.2.3.10 Microscope microscopy*

Observe the different developmental periods of microspore development under 10×, 20× and 40×objectives respectively, and analyze the image acquisition.

### 2.3 Data analysis

Data statistics and analysis were carried out by *Microsoft Excel 2016* software, andvariance and significance were tested by *SPSS 22.0*.

# 3 Results and discussion

## 3.1 Morphological Observation and Measurement of Flower Buds

The results and photographs of the two pea materials are detailed in Table 1 and Figure 1, respectively. In conjunction with Figure 3 and Figure 4, it is clear that the longitudinal diameter of the flower buds from the two pea materials increased progressively with the development of the microspores. There were significant differences in the longitudinal diameter of the flower buds across various sampling intervals, thus allowing for a fast screening of the microspore development stage. However, the transverse diameters and bud aspect ratios showed no significant differences across these sampling intervals

**Table 1** Morphological indicators of different longitudinal diameter intervals of pea buds

| Material name | The bud longitudinal diameter interval/mm | Bud longitudinal diameter/mm | Flower bud transverse diameter/mm | Bud longitudinal diameter transverse diameter ratio |
|---|---|---|---|---|
| YP58 | [2.00,3.00) | 2.77±0.12h | 1.03±0.07e | 2.69±0.13cd |
| | [3.00,4.00) | 3.59±0.18g | 1.20±0.19de | 3.05±0.50bc |
| | [4.00,5.00) | 4.61±0.19f | 1.49±0.23de | 3.14±0.42bc |
| | [5.00,6.00) | 5.60±0.25e | 1.61±0.28de | 3.58±0.77ab |
| | [6.00,7.00) | 6.46±0.34d | 1.70±0.13d | 3.81±0.18a |
| | [7.00,8.00) | 7.58±0.19c | 2.41±0.35c | 3.19±0.42bc |
| | [8.00,9.00) | 8.50±0.19b | 3.03±0.26b | 2.82±0.20cd |
| | [9.00,10.00] | 9.63±0.18a | 4.25±1.07a | 2.39±0.62d |
| YW90 | [2.00,3.00) | 2.43±0.29h | 0.94±0.10e | 2.61±0.38c |
| | [3.00,4.00) | 3.26±0.16g | 1.10±0.16de | 3.01±0.48bc |
| | [4.00,5.00) | 4.42±0.20f | 1.43±0.17cd | 3.13±0.34abc |
| | [5.00,6.00) | 5.44±0.26e | 1.68±0.14c | 3.26±0.29ab |
| | [6.00,7.00) | 6.60±0.14d | 1.76±0.13c | 3.76±0.31a |
| | [7.00,8.00) | 7.65±0.23c | 2.34±0.24b | 3.30±0.36ab |
| | [8.00,9.00) | 8.55±0.19b | 2.55±0.79ab | 3.55±0.80ab |
| | [9.00,10.00] | 9.42±0.26a | 2.93±0.44a | 3.27±0.47ab |

Values are means ± standard errors. Means with the same letter within each column are not significantly different at $p < 0.05$

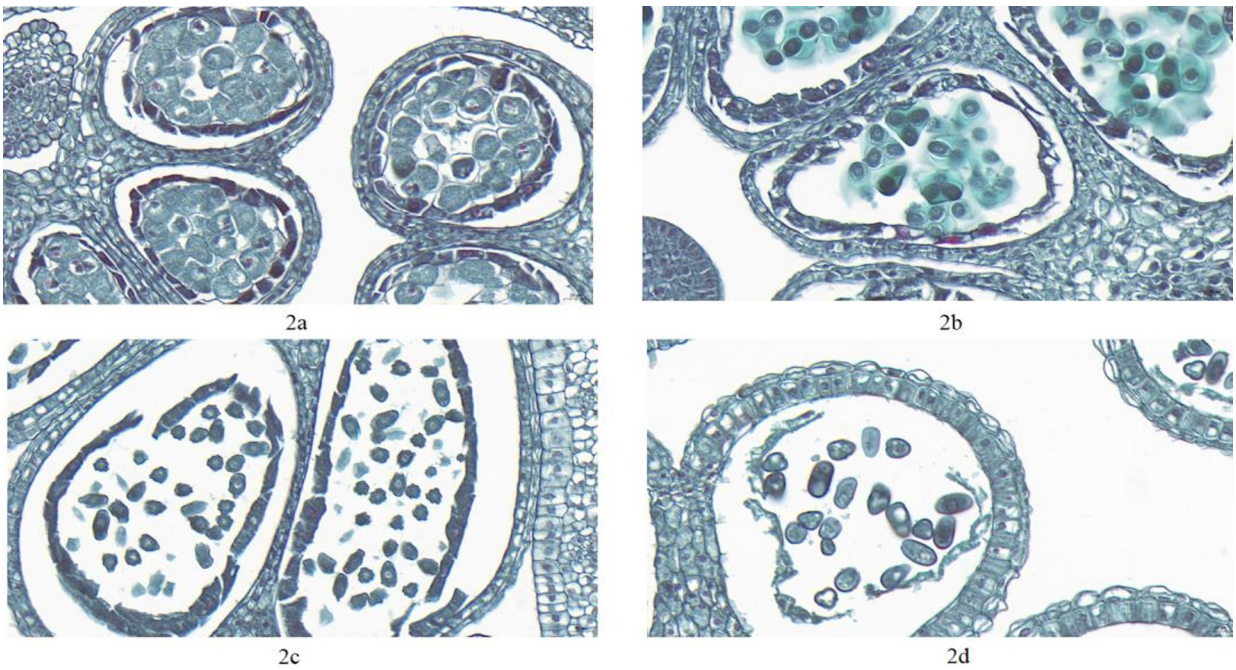

A. YP58 B. YW90

1-8 indicate the interval of longitudinal diameter of flower buds in [2.00,3.00), [3.00,4.00), [4.00,5.00), [5.00,6.00), [6.00,7.00), [7.00,8.00), [8.00,9.00), and [9.00,10.00] mm, respectively.

**Figure 1** 2 buds of pea material

### 3.2 Cytological characteristics of pea microspore developmental periods

Cytological observations showed that the development of microspores of YP58 and YW90 new pea materials mainly went through four developmental periods. The morphological characteristics of different developmental periods of microspores of pea varieties are shown in Figure 2. From Figure 2a, it can be seen that a single large cell with large volume, deep staining and obvious nucleus is the characteristic of microspore mother cell period. After meiosis, the microspore mother cell showed a tetrahedral shape, the callus wall surrounded the four microspores together, i.e. the tetrad period (Figure 2b); the callus wall of the tetrad was disintegrated, releasing free microspores, and finally a single cell was formed, and the nucleus was located in the middle of the cell, the nucleus was large, and the staining was clearly visible, i.e. the mononucleate early to middle stage (Figure 2c); with the advancement of the development, the cell volume continued to increase, and the shape was clearly visible, i.e. the mononucleate early and middle stage ( Figure 2c); the cell volume continued to increase and the staining was clearly visible, and the cell volume continued to increase, and the cell volume increased, and the staining was clearly visible, i.e. the mononucleate early stage. As the development process advances, the cell volume is increasing, the shape is round, the cell vesicles gradually fusion, the formation of large vesicles, sprouting grooves gradually formed, the cytoplasm is thick, rich in organelles, mitochondrial volume is larger, the nucleus is close to the edge of the cell, and therefore is called the mononuclear late stage (Figure 2d). It can be seen that the development of microspores in the two pea materials mainly went through the microspore mother cell period, the tetrad stage, the early-middle stage of the mononuclear stage, and the edge of the mononuclear stage, and each of these developmental periods was characterized by distinctive features.




2a. microspore mother cell 2b. Tetrad stage 2c. Early and middle mononuclear stage 2d. late mononuclear stage
**Figure 2** Cytological characteristics of pea microspore at different developmental stages (×400)

### 3.3 Corresponding relationship between microspore development period and flower organ size in pea

The development of microspores has a certain degree of gradual continuity (i.e., asynchrony), and the same anther often contains microspores of several developmental periods, so we divided the developmental period by the microspores with the highest proportion of a certain developmental stage in the anther as the criterion for judging the developmental period [5]. By observing the microspores of two pea materials in different longitudinal diameter intervals of flower buds, we found that the developmental period of pea microspores was closely related to the size of its longitudinal diameter of flower buds. With the growth of the longitudinal diameter of the flower bud, the development of microspores mainly went through the microspore mother cell period, the tetrad period, the early and middle mononuclear stage, the mononuclear edge stage, and each developmental period was clearly characterized. In the material of YP58, the main stages in Figs. 3a and 3b were the microspore mother cell period, Figs. 3b and 3c were the tetrad period, and Figs. 3e-1h were the early and middle mononuclear stage to the late mononuclear stage. In YW90, Figs. 4a-4c major stages are the microspore mother cell period, 4d major stages are the tetrad period, and 4e-2h major stages are early-mid to late mononuclear stage.

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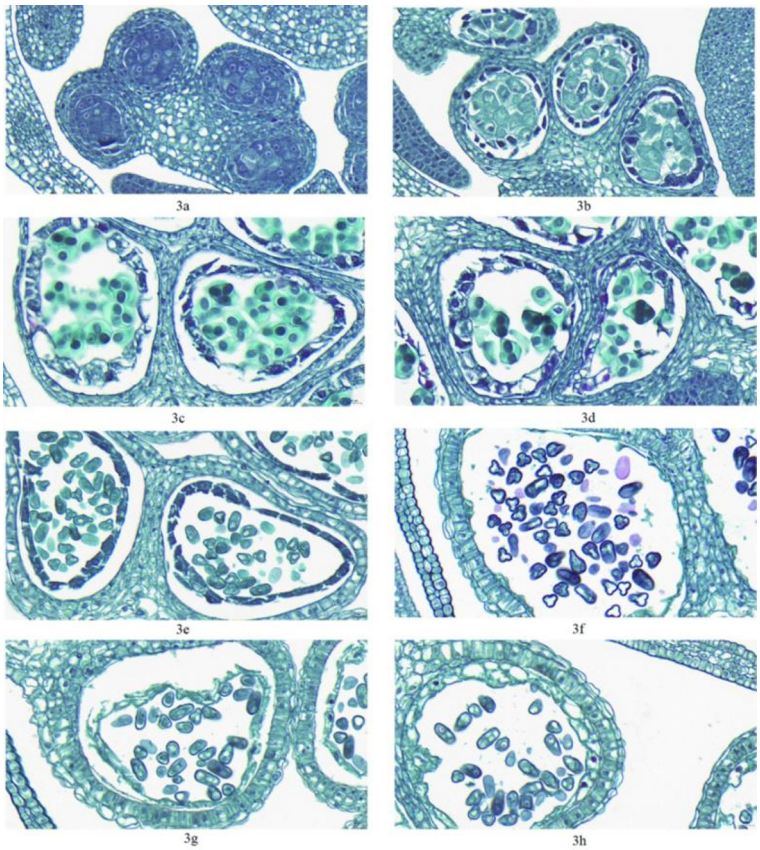
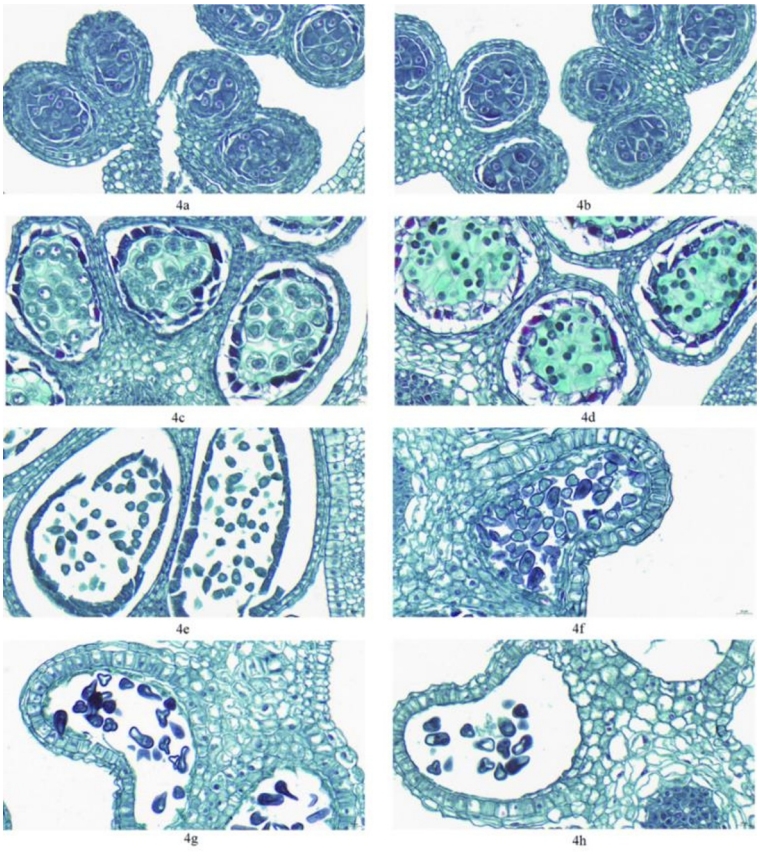



3a-3h indicate the interval of longitudinal diameter of flower buds as [2.00,3.00), [3.00,4.00), [4.00,5.00), [5.00,6.00), [6.00,7.00), [7.00,8.00), [8.00,9.00), and [9.00,10.00] mm, respectively.

**Figure 3** Observation of microspores of YP58 flower buds of different sizes (×400)




4a-4h indicate the interval of longitudinal diameter of flower buds as [2.00,3.00), [3.00,4.00), [4.00,5.00), [5.00,6.00), [6.00,7.00), [7.00,8.00), [8.00,9.00), and [9.00,10.00] mm, respectively.

**Figure 4** Microspore observation of YW90 flower buds of different sizes (×400)

## 4 Conclusion

This investigation meticulously observed the stages of microspore development in pea pollen, identifying distinct characteristics at each phase. The phases included the microspore mother cell period, the tetrad period, the early-mid mononuclear stage, and the late mononuclear stage. A compelling correlation was found between each developmental period and the size of the longitudinal diameter of the flower buds, consistent across two different pea materials. Furthermore, by analysing the anther sections of these two pea materials, the study determined that the microspore mother cell period coincided with a bud diameter of 2.00-4.00mm. The tetrad period was observed when the bud diameter reached 5-6mm, and the mononuclear period commenced when the bud diameter exceeded 6mm.

As there have been no successful reports of pea anther culture, this study serves as a valuable theoretical backdrop for its future development. Potential future research directions include exploring the correlation between the development process of pea microspores and the morphology of flower buds and anthers, considering diverse pea genotypes and planting climates. The ultimate goal is to facilitate direct sampling from the field, optimizing efficiency and saving both time and labor, thereby paving the way for a highly efficient pea anther culture system.

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## References

1. Yang, X.M., Ren, R.Y. (2005) Research progress of pea production and breeding at home and abroad. Gansu Agricultural Science and Technology., 8:3-5. DOI:10.3969/j.issn.1001-1463.2005.08.001. in Chinese
2. Leslie K. (2021) Improved Anther Culture Media for Enhanced Callus Formation and Plant Regeneration in Rice (*Oryza sativa* L.).Plants., 10:839. DOI:10.3390/plants10050839.
3. Willcox, M.C., Reed, S.M., Burns, J.A., Wynne, J.C. (1991) Effect of microspore stage and media on anther culture of peanut (*Arachis hypogaea* L.). Plant Cell Tissue & Organ Culture., 24(1):25-28. DOI:10.1007/BF00044261.
4. Murovec J., Bohanec B. (2012) Haploids and Doubled Haploids in Plant Breeding. InTech., 117(3):200-302. DOI:10.5772/29982.
5. Wang K.B., Wang X.M., Xiong W.W., Fu Long, Kou C.L., Shen X.Y., Cheng G.X., Guo M., Gao Y.M., Li J.J. (2024) Correlation between the developmental period of tomato microspores and floral morphology. Journal of Gansu Agricultural University.,59(01):161-170. DOI:10.13432/j.cnki.jgsau.2024.01.018.in Chinese