Original Article

POLLEN COUNT DYNAMICS IN RAPESEED STAMENS IN EARLY SPRING

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Abstract

Pollen contains vegetative and generative cells that influence the seed set. Accurate pollen assessments help understand the breeding biology of oilseed rape. In order to evaluate the number of pollen grains in a large number of samples, an effective method should be designed. A new method was established to evaluate the pollen counts of five oilseed rape cultivars. This method mainly relies on 5% sucrose + 0.1% agar solution to suspend the pollen and uses a standard curve corresponding to the absorbance and the number of pollen grains to estimate the number of pollen grains. The linear fit formula of this standard curve was precision, and the R^2 value between the pollen count and absorbance reached 0.998. Furthermore, the variation in the pollen counts at three flowering stages and on two kinds of stamens was assessed. The pollen count per flower varied significantly among oilseed rape cultivars and flowering stages (GLM: $p<0.001$). Moreover, the number of pollen grains on long-stamen anthers was significantly higher than that on short-stamen anthers (GLM: $p<0.001$). In conclusion, we established a rapid, accurate method for quantifying pollen grains based on absorbance determined in a liquid suspension by light spectroscopy, which is a feasible method. In addition, the number of pollen grains under different physiological conditions also provides basic data for oilseed rape breeding.

Keywords: *Brassica napus*, oilseed rape, pollen, pollen counting, pollen deposition

INTRODUCTION

Oilseed rape, an important oil crop worldwide (Diepenbrock 2000; Chen et al., 2010), is the most productive oil crop in China and accounts for 57.2% of the total oil production yield in Chinese oil crops (Wang et al., 2019). It is also one of the most popular oil crops in the European Union (Wozniak et al., 2019) and one of the main contributors of vegetable oil in the world (Orlovius 2003). The rapeseed yield depends on successful pollination and the combined fertilization of germ cells, in which fertilization of the ovule by pollen is the most important physiological process (Wang 2011; Müller et al., 2016). Successful fertilization is based on the premise that enough pollen is successfully transferred to the stigma. As an optiomally insect-pollinated crop, the yield and quality of canola depend heavily on the number of pollen grains deposited (Byers 1995; Aizen et al., 2007; Sun et al., 2020; Fairhurst et al., 2021). Inadequate pollen deposition during pollination reduces the number of seeds per pod in oilseed rape (Aizen et al., 2007; Ohnishi et al., 2010; Wang 2011). The number of pollen grains in anthers may be one of the factors that determine the number of pollen grains (Wang et al., 2014), with 3.7% of the pollen in the flower being used for pollination (Schlindwein et al., 2005). Therefore, it is meaningful to develop an efficient and effective method for evaluating the pollen count of oilseed rape to understand pollen dynamics in different

flowering stages and in different cultivars.

The ether-weight method for observing pollen yield was proposed in 1972 and was improved to become a more common method for observing pollen yield (Warakomska, 1972; Szklanowska, 1995). For this method, the anther is rinsed with pure ether and ethanol, and the pollen residues in the anthers are examined under microscope. Moreover, a particle counter was used to measure the number of pollen grains (Thomson et al., 2001; Denisow et al., 2022). However, this method often includes dust and other small debris, which require cumbersome later calculation to increase the accuracy of the data. In addition, more accurate particle counters are expensive and not easy to use in field trials. There are many existing methods for pollen counting, among which the most widely used involves placing anthers in a sodium hexametaphosphate solution or distilled water (Ye et al., 2010; Wang et al., 2017) and then adding 1% cellulase (Du et al., 2015; Zhu et al., 2016) or other enzymes for digestion. Afterwards, the pollen suspension is shaken to disperse the pollen, and the pollen count is determined under a microscope with a blood cell counting plate (Godini 1981; Wu et al., 2017). However, this method is labor intensive (Comtois et al., 1999; Carinanos et al., 2000; Kakui et al., 2020), so spectroscopic measurements have emerged as an alternative approach in which pollen counts are measured based on absorbance (Wizenberg et al., 2020). Such measurements have been demonstrated to be efficient and effective for counting pollen.

Due to the influence of intrinsic genetic characteristics (Sidhu 2019; Zhang et al., 2020), the pollen count differs among oilseed rape cultivars (Ye et al., 2010; Du et al., 2015; Fairhurst et al., 2021). A male sterile line of *Brassica napus* was found to produce very little pollen and showed no pollen viability (Hu et al., 2023). Furthermore, the pollen count of a single flower in cabbage cultivars has been found to range from 20,000 to 858,000 grains (Sun et al., 2020). It has also been demonstrated that the pollen counts of linden trees vary dynamically among different flower stages, with the highest pollen count occurring at the anthesis stage (Weryszko-Chmielewska et al., 2010). In addition, the number of pollen Brassicaceae grains also changed (Masierowska, 2012). Each oilseed rape flower has two short and four long stamens, and the difference in stamen length has been indicated to be related to the interaction between the plants and pollinating insects (Oliveira et al., 2022). Is this difference associated with the difference in pollen counts on the stamens? In this context, it is meaningful to explore the pollen count variation characteristics of oilseed rape.

In order to understand the dynamics of pollen grains in different rape cultivars at different flowering stages and stamens, there must be an effective method that can detect the number of pollen grains quickly. A suitable method based on hematometer and the absorbance of spectrophotometer would measure the number of pollen grains through the use of the standard curve between the pollen grains and absorbance. Here, we optimize a suitable solution that can suspend the pollen particles evenly and establish a standard curve to estimate the pollen grains of oilseed rape. In addition, changes in pollen grain numbers at the different cultivars, the flowering stages, the long and short stamens were evaluated. These results could be used to guide oilseed rape breeding.

MATERIAL AND METHODS

Oilseed rape materials and sampling

The experiment was conducted in Luoping County, Yunnan Province, which is the earliest oilseed rape flowering area in China. Five oilseed rape cultivars - Aiyouwang (AYW), Hengheyou998 (HHY998), Ningde21 (ND21), Dehuiyou (DHY) and Huayou (HYXL) were planted in ten plots in October 2020. Samples were collected at three flowering stages -initial flowering, full flowering and final flowering, to compare the pollen grain differences among stamens in spring 2021. Thirty flowers were collected from each plot. It took twenty days from the first sampling to the sampling deadline. The average low temperature was 9.57°C, the average high temperature was 18.36°C, and the precipitation was 1406.8 mm at the experimental field. Initial flowering was defined as the time at which approximately 25% of the plants started

flowering; full flowering was defined as the period when more than half of the main branches and 2/3 of the branches in the plot plants showed flower opening; and final flowering was the period when 75% of the inflorescences senesced (Fang et al., 2019).

Twelve oilseed rape plants were randomly selected in each plot, and three large flower buds were picked from each plant. The flower buds were directly placed into Eppendorf tubes. The stamens were removed with forceps before the anthers split. Each stamen was kept separately in a 1.5 ml Eppendorf tube. The flowers with collected stamens need to meet the following criteria: there are four long stamens and two short stamens in the flowers, and the stamens are normally developed. Withered stamens were discarded. The stamens of thirty flowers were sampled.

Suitable suspension solution selection

A high-concentration pollen suspension was prepared by adding 15 ml distilled water and 0.5 g pollen to a centrifuge tube and subjecting the pollen to ultrasonic disruption (JY92-IIDN ultrasonic cell homogenizer, Ningbo Xinzhi Biotechnology Co., Ltd, Ningbo, China) to disperse pollen clumps. The parameters of the ultrasonic disruptor were horn Φ 6, power 135-650 W, ultrasound 8 s, interval 2 s, and working time 2 min.

Four kinds of suspension solutions were prepared: 20% sodium hexametaphosphate (Wang et al., 2013), 5% sucrose + 0.1% agar (Chen et al., 2014), and 10% sucrose + 0.05% agar (Lei et al., 2020), and distilled water in a final volume of 100 mL.

Ten milliliters of each suspension solution were placed in a 15 ml centrifuge tube. Then, 150 µl of the high-concentration pollen suspension was added to each tube, and mixing was performed. Five microliters of the pollen suspension solution were sampled from 1 cm below the liquid surface three times at 0, 20, 40, 60, 80, and 100 min. The number of pollen grains was counted in the white blood cell measurement area of microscope blood cell counting board.

After 20 min, 5 µl of the pollen suspension solution was sampled at a depth of 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, and 4.5 cm below the liquid surface three times. Sampling conducted from shallow to deep. The number of pollen grains was counted in the white blood cell measurement area of a microscope blood cell counting board. Distilled water was used as the control. Finally, it 5% sucrose + 0.1% agar solution was found to be the most suitable solution and was used for the subsequent experiments.

Preparation of standard curves

A suspension solution of 5% sucrose + 0.1% agar was prepared. A high-concentration pollen suspension was prepared by adding 15 ml of the suspension solution and 0.5 g pollen to a centrifuge tube. An ultrasonic disruptor was used to disperse the pollen clumps. The acoustic disruptor parameters were: amplicon Φ 6, power 210 W, sonication 8 s, and interval 2 s. High-concentration pollen suspensions (0, 50, 100, 150, 200, 250 and 300 µl) were added to the 5% sucrose + 0.1% agar suspension solution in a final volume of 10 ml. The pollen suspension solutions were mixed with the vortex for 2 min.

The prepared pollen suspensions were placed into a spectrophotometer (752 UV VIS spectrophotometer, Shanghai Jinghua Technology Instrument Co., Ltd., Shanghai, China), and the absorbance was measured at a wavelength of 425 nm with a pure 5% sucrose + 0.1% agar solution as the blank control. The assessment of each sample was replicated three times, and the average value was taken as the final result. Aliquots of 5 µl of the sample solutions with different pollen concentrations were dropped onto a hemocytometry plate (Hemocytometer, Shanghai Qiujing Biochemical Reagent Instrument Co., Ltd., Shanghai, China) and observed under a microscope to count pollen, which was performed five times. The mean was taken as the final result, which was calculated as number of pollen grains = $(N/4) \times 10^5$, where N is the number of pollen grains observed microscopically.

Sample absorbance was plotted against pollen counts, and the data were linearly fitted using R project version 3.6.2 to obtain a plot of pollen quantity versus absorbance.

Sample pollen count measurements

The most suitable suspension solution (5% sucrose + 0.1% agar) was prepared. Pollen samples were placed in 10 ml of the suspension solution and fragmented with an ultrasonic disruptor. The horn setting of the ultrasonic disruptor was Φ 6, the power was adjusted to 210 W, the ultrasound application time was 8 s, and the interval was 2 s. The absorbance of the sample solution was measured at a wavelength of 425 nm. A total of 900 samples from five cultivars were measured with 180 samples from each cultivar.

Data analysis

The pollen grains in different suspensions do not fit the normal distribution. To test stabilization by different suspension solutions, we performed nonparametric tests to determine the differences in pollen counts at different times after mixing and different depths from the surface of the suspension solution. A linear regression model was used to test the fit between the absorbance and pollen count measurements, where the R^2 value represented the accuracy of the absorbance and pollen counts. Generalized linear models were used to compare pollen counts among different cultivars, long and short stamens and different bloom stages. All data were analyzed and plotted using R project version 3.6.2.

RESULTS

Suspension solution selection

The speed of pollen deposition was significantly different among the four suspension solutions (Kruskal-Wallis H: $p<0.001$). With increasing time, the number of pollen grains at a depth of 1 cm under the surface differed significantly among the four suspension solutions (Kruskal-Wallis H: $p<0.001$). The suspension solutions of 5% sucrose + 0.1% agar and 10% sucrose + 0.05% agar were much more stable than distilled water or the 20% hexametaphosphoric acid solution at different times (Fig. 1). However, pollen deposition in the 5% sucrose + 0.1% agar and 10% sucrose + 0.05% agar suspension solutions showed a slight decrease after 30 min. The average numbers of pollen grains in the 10% sucrose + 0.05% agar solution, 20% sodium hexametaphosphate, the 5% sucrose + 0.1% agar solution and distilled water were 10.52±0.29 (n=216), 18.15±0.91 (n=216), 8.79±0.28 (n=216) and 4.43±0.38 (n=216), respectively.

As the depth of the suspension solution increased, the numbers of pollen grains deposited after 5 min differed significantly among the four suspension solutions (Kruskal-Wallis H: $p<0.001$). The pollen grains at different depths were much more stable in the 5% sucrose + 0.1% agar and 10% sucrose + 0.05% agar suspension solutions than in distilled water and the 20% hexametaphosphoric acid solution (Fig. 2). The average numbers of pollen grains in the 10% sucrose + 0.05% agar solution, 20% sodium hexametaphosphate, the 5% sucrose + 0.1% agar solution and distilled water were 11.44±0.43 (n=216), 16.40±1.16 (n=216), 9.78±0.37 (n=216) and 10.38±0.63 (n=216), respectively.

Establishment of standard curves

According to the stability test, the suspension solution of 5% sucrose + 0.1% agar was the most suitable solution. The relationship between the absorbance and the amount of pollen was established based on the number of pollen grains counted with the use of a microscope (Fig. 3). The calculation applied to the pollen count and the absorbance was $y = 109991x-2647.47$, where y is pollen count and x absorbance ($R^2 = 0.998$).

Pollen count variation in oilseed rape

There were significant differences in the total counts of pollen among different varieties at different flowering stages (GLM: $p<0.001$, Tab. 1). Among the five cultivars, the ND21 cultivar had the highest total pollen grains per anther count [42388.14±1095.03 grains (n=90)], followed by the DHY cultivar [38460.46±1486.87 grains (n=87)], and the pollen count of these two cultivars was significantly higher than those of the other three cultivars (Fig. 4). The lowest total pollen count was found in the AYW cultivar [31625.22±1202.70 grains (n=90)].

Among the three flowering stages, the full bloom stage showed the highest pollen count, and the late flowering stage showed the lowest (Tab. 1).

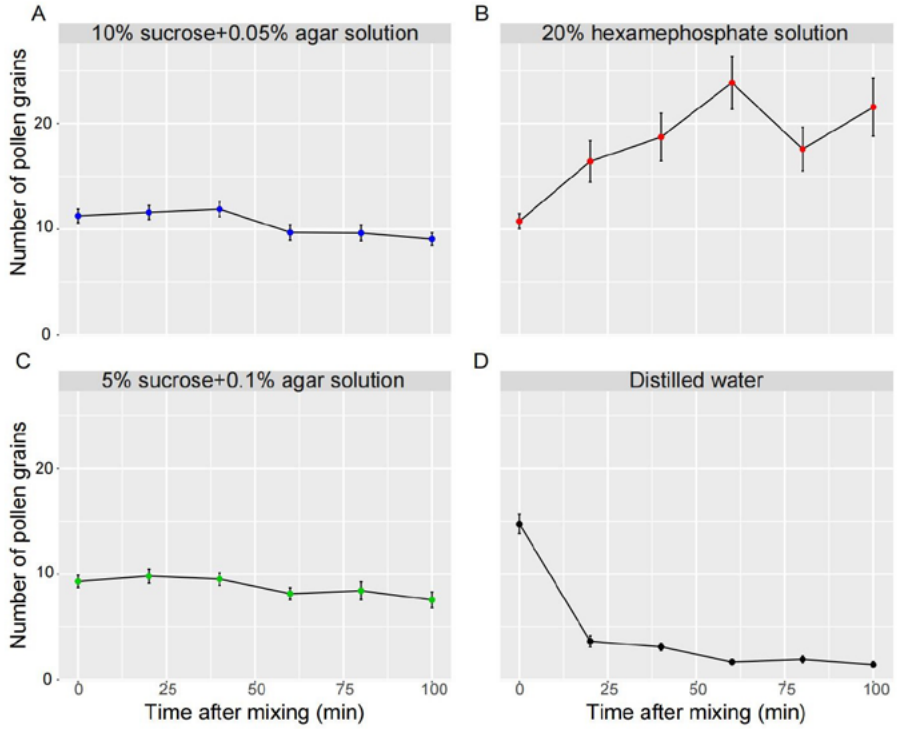

Fig. 1. The number of pollen grains in 5 µl of a suspension solution pipetted from a 1-cm depth below the liquid surface was counted by hemocytometry after different standing times in four suspension solutions (A. 10% sucrose + 0.05% agar solution, B. 20% sodium hexametaphosphate solution, C. 5% sucrose + 0.1% agar solution and D. distilled water). Data are presented as the means ± SEs.

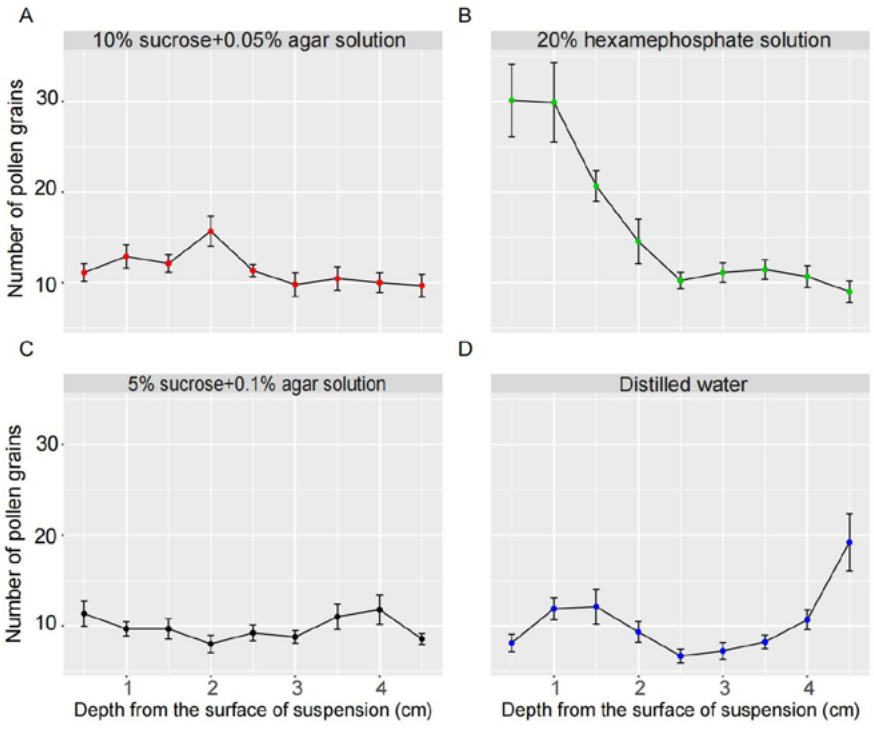

Fig. 2. The number of pollen grains in 5 µl of a suspension solution pipetted from different depths below the liquid surface was counted by hemocytometry in four suspension solutions (A. 10% sucrose + 0.05% agar solution, B. 20% sodium hexametaphosphate solution, C. 5% sucrose + 0.1% agar solution and D. distilled water). Data are presented as the means ± SEs.

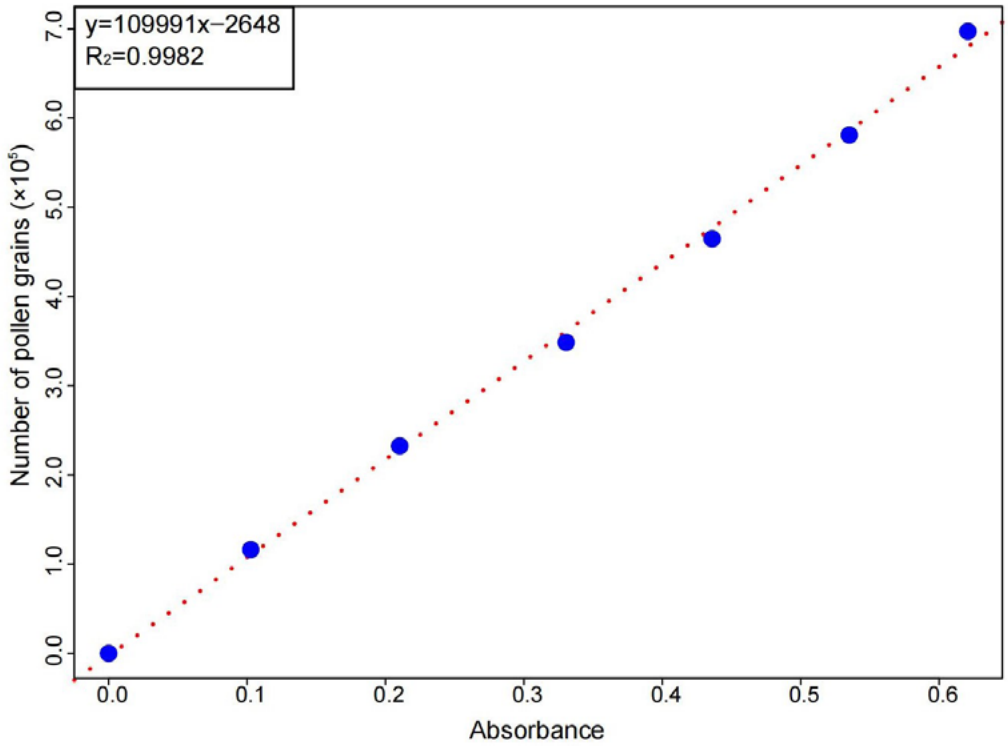

Fig. 3. The linear relationship between the number of pollen grains in 5 µl of a 5% sucrose + 0.1% agar suspension solution assessed by hemocytometry under microscopy and the absorbance measured by light spectroscopy at a wavelength of 425 nm.

Table 1.

Number of pollen grains per flower in five oilseed rape cultivars

Cultivar	Initial flowering stage	Full bloom stage	Final flowering stage
AYW	180524.23 ± 12083.10^{c}	252715.64 ± 7947.12^{bc}	136014.13 ± 5573.03^{c}
HHY998	181954.12 ± 14039.44^{c}	242706.37 ± 9721.72^{c}	147306.64 ± 7737.18^{bc}
ND21	276730.56 ± 12619.33^{a}	270571.01 ± 8632.42^{b}	215685.00 ± 9261.30^{a}
DHY	217958.17 ± 13535.64^{b}	314539.30 ± 9816.28^{a}	168168.46 ± 8056.94^{b}
HYXL	175464.60 ± 9152.36^{c}	270131.04 ± 7004.21^{b}	160029.05 ± 6928.05^{bc}
P value	0.00	0.00	0.00

Data are presented as the means ± SEs. Identical lowercase letters indicate no significant difference ($p>0.05$); different lowercase letters indicate a significant difference ($p<0.01$).

At the early flowering stage, the pollen counts among different varieties were significantly different (GLM: $p<0.001$). The pollen count of the ND21 cultivar was highest on both long anthers [48782.96±2232.10 grains (n=90)] and short anthers [40799.37±2866.30 grains (n=90)], where the counts were significantly greater than those of the other cultivars (GLM: $p<0.001$, $p=0.002$). The pollen count of the AYW cultivar was lowest on both long anthers [32669.13±1377.21 grains (n=90)] and short anthers [29537.41±1315.39 grains (n=90)] among all cultivars (GLM: long

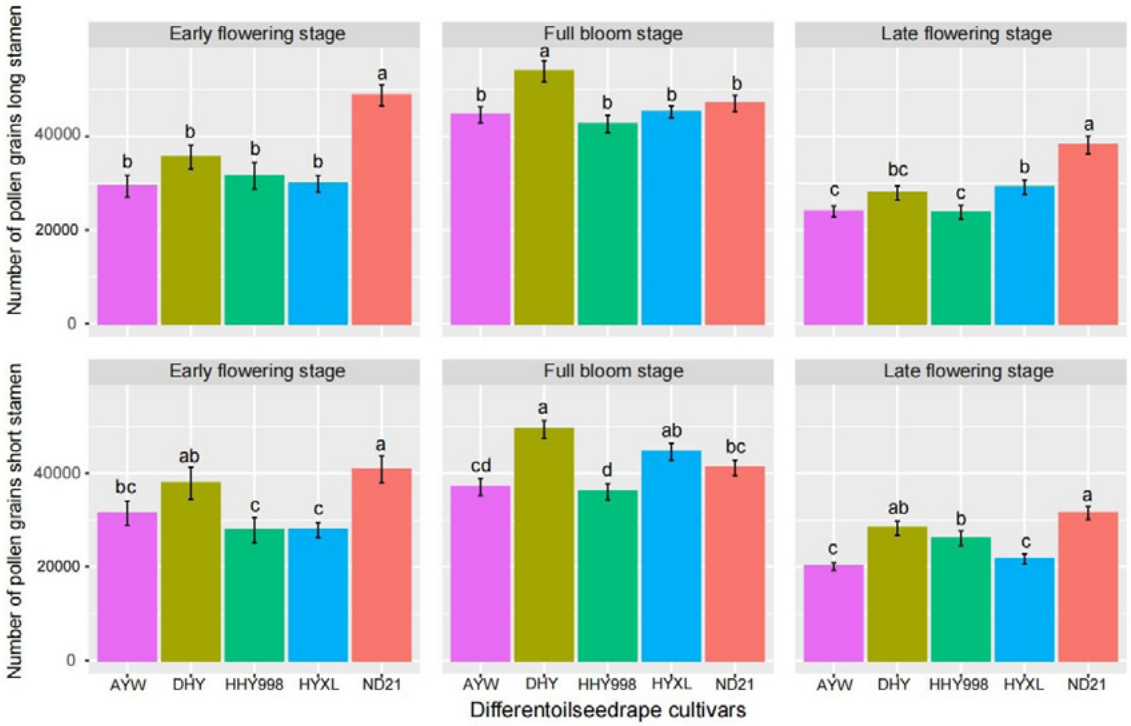

Fig. 4. Pollen count per anther at different flowering stages and on stamens of different lengths among five oilseed rape cultivars. Data are presented as the means ± SEs. Identical lowercase letters indicate no significant difference (p>0.05); different lowercase letters indicate a significant difference (p<0.05).

anthers p=0.279, short anthers p=0.937).

During the full bloom stage, the DHY cultivar presented the highest pollen count, where the average pollen counts of the long and short stamens were 53937.30±2238.49 (n=87) grains and 49395.04±1883.32 (n=87) grains, respectively, which were significantly higher than the counts of the other cultivars (GLM: p<0.001). There was no significant difference in the long anther pollen counts among the AWY, HHY98, ND21 and HYXL cultivars.

At the late flowering stage, the pollen count of the ND21 cultivar was significantly higher than those of the other cultivars, with 38168.73±1890.453 (n=90) grains on long stamens and 1505.05 ±1373.28 (n=90) grains on short stamens. The pollen counts of the long stamens were not significantly different among the AYW, DHY and HHY998 cultivars (GLM: p=0.158).

DISCUSSION

When the pollination media are consistent, the quantity and quality of insect-pollinated crop fruits are limited by the number of effective pollen grains to some extent, which may indirectly affect the fertilization of flowers. Flowers with a large amount of pollen can be collected by pollinators many times and provide more opportunities for stigmas, which may indirectly affect the fertilization (Wilson et al., 1991; Földesi et al., 2021). Moreover, the pollen count affects the crop pollination efficiency (Ilgin et al., 2007; Földesi et al., 2021). Therefore, it is necessary to select crop cultivars with high pollen counts to benefit fertilization and improve the attraction of pollinators. Here, we established an effective and efficient method for determining the pollen count of oilseed rape. Based on the developed pollen counting method, the pollen counts of different oilseed rape cultivars, long and short stamens, and different flower stages were found to be significantly different. The pollen count per flower varied significantly among oilseed rape cultivars. The pollen counts also varied with the flowering dynamics. Furthermore, the pollen count of the long stamens was higher than that of the short stamens. These results can provide guides for

the breeding of oilseed rape to select high-yield cultivars.

The traditional pollen counting method relies on microscopy-based observations and the standard curve between the light spectrum and pollen count (Wizenberg et al., 2020). Here, we optimized an oilseed rape pollen counting procedure and identified a suitable suspension solution of 5% sucrose + 0.1% agar (Chen et al., 2014; Lei et al., 2020). Lei et al. (Lei et al., 2020) compared the distribution status of pollen in 0.05%-0.15% sucrose agar solutions and found that the stability of pollen suspensions was stable in 10% sucrose + 0.05% agar. The agar concentration used by those authors was lower than that selected in the present study, but their sucrose content was higher. Therefore, the sugar and agar concentrations can be modulated to acquire a suitable pollen suspension solution. The experiment confirmed that the stability of rapeseed pollen suspension in agar solution is suitable, because due to the certain coagulability of agar solution, it should optimize the suspension solution to different plant pollen grains.

The R^2 value of the fitted curve between the pollen count and absorbance reached 0.998, which indicates that the accuracy of our prediction procedure was extremely high and that it could be used to evaluate the pollen count of oilseed rape. The correlation between the pollen count and spectral readings has also been reported in *Cannabis sativa,* where the R^2 value is just 0.86 (Wizenberg et al., 2020). The developed suspension and measurement procedure could provide an efficient and effective method for oilseed rape pollen counting.

The amount of pollen varied greatly among the five oilseed rape cultivars. The cultivars of ND21 showed higher pollen counts than the other four cultivars. It has been reported that oilseed rape pollen counts are affected by genetics (Vonhof et al., 1995; Campos et al., 2021; Kakui et al., 2021) and environmental factors (Hedhly et al., 2005; Jäger et al., 2008; Chaturvedi et al., 2021). A male-sterile line of *Brassica napus* was found to produce very little pollen and showed no pollen viability, which was also the case in a cytoplasmic male-sterile line with trace pollen (Hou et al., 1991; Hu et al., 2023).

Moreover, it was found that the pollen counts in different flowering periods were significantly different. The five studied oilseed rape cultivars showed similar trends, with higher pollen counts in the full bloom stage than in the initial and late flowering stages. It has been proved that the highest pollen number of linden trees is the full flowering stage, which was consistent with the results in this paper (Weryszko-Chmielewska et al., 2010). The high pollen count in the full bloom stage implied that there may be higher reproductive pressure in this stage. From the perspective of insect pollination, a high pollen count should provide a greater reward to pollinators to improve the pollination competition (Oliveira et al., 2022).

An interesting finding in this study was that the pollen counts of the long stamens were greater than those of the short stamens. In the studied oilseed rape cultivars, the long stamens exhibited higher pollen counts than the short stamens, except in the AYW cultivar in the early flowering stage and HHY998 in the late stage. Variations among stamens and styles have been reported to be an outcome of selective forces, driven mainly by pollinators (Oliveira et al., 2022). However, we have not obtained any information indicating why the long and short stamens show different pollen counts, and therefore further studies should be performed to explore the functions of the long and short stamens.

Overall, our study evaluated different suspension buffers to identify a stable and efficient method for counting the pollen of oilseed rape. A suspension solution of 5% sucrose + 0.1% agar was found to be the most uniform solution among the four tested buffers and produced stable and accurate results according to absorbance with light spectroscopy measurements. This high-efficiency pollen counting method could be used to understand the pollen-related biology of oilseed rape. It is especially useful for pollen counting in a large number of samples. Furthermore, our results indicated that the pollen counts of different oilseed rape cultivars, long and short anthers, and different bloom stages

were significantly different. This characteristic of oilseed rape biology should be considered by breeding and pollination services to improve the resultant oilseed rape harvest. Moreover, the differences in the pollen amounts of long and short anthers indicated that the functions of different stamens. Although samples were only collected from five rape cultivars in our study, the pollen count of oilseed rape was affected obviously by different oilseed rape cultivars, physiological states, and morphological characteristics. To further improve the control of pollen dynamics in oilseed rape, pollen release should be performed at different bloom times.

Declaration of competing interest

The authors declare no conflict of interest.

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