RESEARCH ARTICLE Legume Research- An International Journal, Volume Issue : ()

Influence of Sucrose, Boric Acid and Calcium Chloride on *in vitro* Pollen Germination and Tube Elongation of Alfalfa

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10.18805/LRF-749

ABSTRACT

Background: In a genetic breeding program, understanding the viability of pollen germination and pollen tube growth is an essential factor for artificial hybridization. Currently, little is known about the influence of sucrose, boric acid concentrations and calcium chloride on pollen viability in alfalfa. This study is valuable for providing information about pollen germination requirements to support further improvement of artificial pollination and breeding programs in alfalfa.

Methods: The single-factor experimental and an orthogonal design method (L_{16} $[4]^3$) was used to compare the effectiveness of key factors (*e.g.*, concentrations of sucrose, boric acid and calcium chloride) to determine the pollen vigor of *Medicago varia* Martin. 'Caoyuan No.1'and *M. ruthenica* L. Trautv. 'zhilixing' and to optimize the culture medium for *in vitro* pollen germination. Four different varieties were chosen to compare the diurnal variation of pollen vigor.

Result: The four factors that influenced pollen germination in *Medicago varia* Martin. 'Caoyuan No.1'and *M. ruthenica* L. Trautv. 'zhilixing' in order of significance are as follows: sucrose > H_3BO_3 > $CaCl_2$. These variables influenced pollen tube growth in the following order: sucrose > H_3BO_3 > $CaCl_2$. The optimal culture medium for promoting pollen vigor of *Medicago varia* Martin.'Caoyuan No.1' was 10% sucrose, 300 mon/L H_3BO_3 and 100 mon/L $CaCl_2$; that of *M. ruthenica* L. Trautv. 'zhilixing' was 10% sucrose, 200 mon/L H_3BO_3 and 50 mon/L $CaCl_2$. Among several species of alfalfa, 'Caoyuan No.1' shows the highest pollen vigor and the daily pollen activity curves of all varieties showed a single peak type.

Key words: Alfalfa, Sucrose, Boric acid, Calcium chloride, Pollen Germination, Tube growth.

INTRODUCTION

Alfalfa (*Medicago sativa* L.), as an essential legume forage in the world, has the characteristics of high grass yield, rich nutritional value, strong adaptability and palatability and cutting resistance. It also plays an active role in ecological management such as soil improvement, water storage and fertilizer conservation. It was a strictly cross-pollinated plant with a low self-fertilization rate. Studying the process of sexual reproduction is of great significance for improving the seed-setting rate of alfalfa. Cross-breeding is an important way to cultivate high-quality and high-yielding alfalfa. Commercial production of hybrids obtained from interspecific and intraspecific crosses can achieve better heterosis. Pollination is one of the most important factors in successful hybridization, but its process is complex and depends on many factors, such as rainfall, humidity and wind which can affect this phenomenon in different ways.

As a manifestation of plant germplasm, pollen plays an irreplaceable role in plant genetic breeding and germplasm resource research and the level of pollen vigor in artificial pollinations directly affects the success or failure of plant hybridization. There are many ways to check the pollen viability; for example, I2-KI and TTC staining and *in vitro* pollen germination are common methods. However, different species often use different methods to detect the pollen germination and pollen viability. A study by Melloni *et al.* (2013) demonstrated that lactophenol blue staining was more sensible than the iodine staining method to detect pollen viability of sugarcane. There are also studies (Heidmann *et al*., 2016) showing that impedance flow cytometry (IFC) determines the electrical properties of cells by using microfluidic chips and analyzes a high throughput of pollen cells for viability screening in a standardized method, which is widely used in many species such as wheat, corn, hazelnut, tomato, tobacco, *etc*. (Rafiq *et al.*, 2021). At present, most scholars believe that in vitro pollen germination is the most common and simplest method and can be applied to study many basic and applied aspects of pollen biology (Gomez-Mena *et al*., 2022). This technique is more suitable for detecting viable and potential pollen form cross-pollinated plants (Schueler *et al*., 2004). Several

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How to cite this article: Shi, R., Shi, F. and Wang, S. (2023). Influence of Sucrose, Boric Acid and Calcium Chloride on *in vitro* Pollen Germination and Tube Elongation of Alfalfa. Legume Research. doi:10.18805/LRF-749.

Submitted: 05-04-2023 **Accepted:** 19-05-2023 **Online**: 02-06-2023

extrinsic and intrinsic factors impact pollen germination and pollen tube growth. Organic and inorganic substances such as sucrose, boric acid and calcium nitrate need to be added to the culture medium for in vitro pollen development. (Batos and Miljković, 2019) (Beck-Pay, 2012). The most common external factors include incubation time, incubation temperature and storage temperature (Báez *et al*., 2002; Wang *et al*., 2021). Many plant biologists and researchers suggested that each species required different growth conditions and culture media. Tuinstra and Wedel *et* al. (2014) identified that *Sorghum bicolor* (L.) Moench reached maximum germination *in vitro* on agar medium with 0.9 M sucrose, 2.43 mM boric acid and 2.12 mM calcium nitrate. Li *et al*. (2003) found that a low temperature of 0-2°C was the best condition for storage of pollen of syringa. Recently, an increasing trend in publication was seen in the flowering characteristics and genetic variation of alfalfa research. The release of genome and transcriptome versions has promoted surveys of flowering-related gene content and comparative phylogenetic analyses of gene families (Burgarella *et al*., 2016; He *et al*., 2022). On the other hand, the effects of time, temperature and plant variety on alfalfa pollen viability are preliminarily discussed, which found that time since removal from the anthers was the most prevalent factor affecting pollen viability in alfalfa, did not vary among plant varieties or different temperatures (Brunet *et al*., 2019). However, little research has been done on ensuring the most suitable media selection for determining pollen viability. Therefore, the purpose of this study was to explore the effects of sucrose, boric acid and calcium chloride on pollen germination and pollen tube growth in alfalfa and identify the optimal conditions for in vitro pollen germination.

MATERIALS AND METHODS

The study was carried out in 2022 at the Inner Mongolia Agricultural University Science Building, in China. Different research materials were propagated by cuttings in seedling trays, those seedlings with developed cotyledon leaves and strong roots were transplanted to an experimental farm and natural conditions and management practices were consistent. The site is located in the central part of the Inner Mongolia Autonomous Region (Latitude 40°83′ and Longitude 111°73). The climate is typical of continental climate, with an average annual precipitation of 400 mm. The morphological characteristics of the four materials used for the pollen germination test are shown in Table 1.

The flowers were collected at around 9 a.m. during the full flowering period and stored on ice in an ice box at 0-4°C. The sample was asked to be brought it to the laboratory as quickly as possible. Pollen culture adopts a solid culture method with a 1% agar concentration and pH adjusted to 6.0. The effects of sucrose, boric acid and calcium chloride on pollen germination were determined by single-factor experiments. A total of three treatments were set up in the experiments, with five replicates per treatment. The sucrose experimental group contained a total of 5 different culture media with different concentrations of sucrose (2%, 5%, 10%, 15% and 20%). The mass concentration of boric acid was fixed at 4 levels: 100 mg/L, 150 mg/L, 200 mg/L and 300 mg/L. The apparent concentration of calcium chloride was adjusted to six levels: 50 mg/L, 100 mg/L, 150 mg/L and 200 mg/L. Furthermore, the control group was without the corresponding substance. All treated media were heated and poured onto a concave slide. Then the pollen grains to be tested are distributed on the surface of the cooling medium, after incubation at 25°C for 40 minutes, five fields of view were randomly selected under the microscope to take pictures and each field included no less than 50 pollen grains. Pollen germination is considered to be the length of the pollen tube beyond the diameter of the pollen grain. The microscopic imaging system software was used to measure the length of pollen tubes germinated by pollen grains in the field and the average germination rate and pollen tube length were calculated statistically. Single-factor analysis was performed to determine the effect of the concentration of sucrose, boric acid and calcium chloride on pollen germination in alfalfa and lucerne. An orthogonal experimental design (L_{16} $[4]^3$) was applied to determine the best combination of in vitro pollen germination experimental conditions. L_{16} $[4]^3$ indicates three factors, four levels and sixteen trials. Three factors are sucrose (A), boric acid (B) and calcium chloride (C). Each trial was repeated 3 times. Table 2 shows the experimental design. The ideal medium was used to measure the pollen viability of different varieties and every 2 hours for three consecutive days from 08:00 to 18:00.

For each treatment, at least 50 pollen grains and pollen tubes were randomly chosen for germination frequency calculation and pollen tube length measurements. Calculation of pollen germination based on pollen tube length exceeding pollen diameter.SAS software was used to test the significance of the difference and the Tukey method was used for multiple comparison analyses.

$$\text{Germination rate} = \frac{\text{Germinated pollen}}{\text{Total number of pollen grains}} \times 100$$

RESULTS AND DISCUSSION

Effects of sucrose concentrations on pollen germination and tube growth

During pollen germination, sucrose not only provides nutrients and carbon sources, but also plays a role in regulating environmental osmotic pressure (Jia *et al*., 2022). It was verified that CY and ZB showed a trend of rising first and then falling in the germination percentage of pollen grains and pollen tube growth with varying sucrose concentrations (Fig 1A,B). A suitable concentration of sucrose has a great influence on both germination and pollen tube development. A low concentration of sucrose can lead to a lack of energy; whereas a high concentration of sucrose will damage the pollen cells. When the sucrose concentration of both was 5%, the germination percentage of pollen grains and pollen tube growth reached the highest level, but above 10% can inhibit the germination of pollen grains. Numerous studies showed significant differences between different species and between the same species across different growth conditions. The reason may be the flowering state and catkin maturity level at the time of sampling.

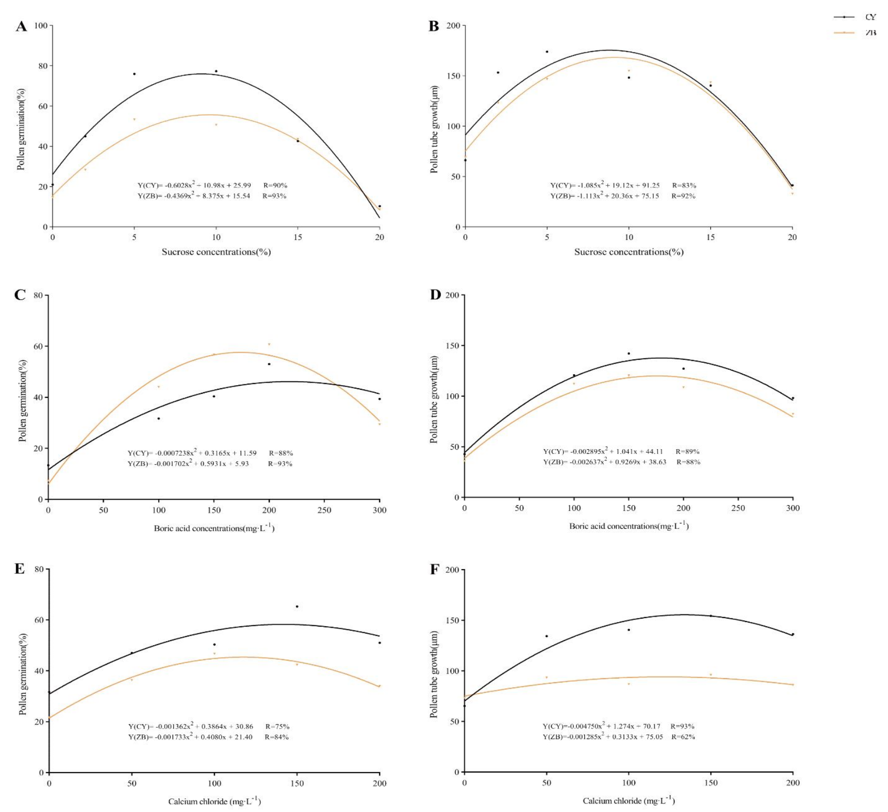
Effects of boric acid concentrations on pollen germination and tube growth

At present, there are many research reports on the effects of boric acid on pollen germination and tube growth. Boric acid is essential for pollen germination, pollen tube growth and pollen tube guidance. In vitro pollen germination tests on a germination medium in the absence of boric acid showed defects in pollen tube growth, as has been demonstrated on species such as Annona cherimola Mill, (Di Giorgio *et al*, 2016) *Malus domestica* L., (Sharafi and Raina, 2020), Lilium logiflorum (Dickinson, 1978). Boric ions can form complexes with sucrose, so that sugar can easily enter the tissue through the plasma membrane, thereby promoting the absorption and metabolism of sugar. Boric can also promote the synthesis of pectin, which is beneficial to the construction of pollen tube walls (Fang *et al*, 2016).

The results of the analysis (Fig 1D) proved that the average pollen tube length of Caoyuan No.1 pollen was about 127 μm and maximum germination was 53% in the concentration of 200 mg/L and the average pollen tube length of Zhilixing pollen was about 120 μm in the concentration of 150 mg/L and maximum germination of 60% in the concentration of 200 mg/L. Boric acid concentration can significantly increase the pollen germination rate in

Table 1: Materials used for the crossbreeding experiment.

Variety	Code	Flower colour	Characteristics
M. ruthenica L. Sojak ‘zhilixing’.	ZB	Yellow/purple	Upright stem strong resistance
M. falcata L.	HH	Yellow	The main thick tap root
M. sativa L. ‘Xinjiang Daye’	XJ	Purple	Large leaves
M. varia Martin. ‘Caoyuan No.1’	CY	Light green	Corolla multicolor



‘Caoyuan No. 1’ (CY) and *M. ruthenica* L. Trautv. ‘zhilixing’ (ZB) pollen grains in culture media containing different concentrations of different concentrations of sucrose (A, B), boric acid (C, D) and calcium chloride (E, F).

Fig 1: Pollen germination rate and pollen tube growth of *Medicago varia* Martin.

Caoyuan No.1 and Zhilixing (Fig 1C), but not always positively correlated. When the concentration of boric acid reaches a certain value, the length of the pollen tube and germination begin to decline. These findings are consistent with the results obtained by Wang *et al*. (2003).

Effects of different calcium chloride on pollen germination and tube growth

Calcium ions was one of the essential elements for pollen tube germination in the in vitro pollen germination test, which was confirmed for 86 species in 39 plant families (Brewbaker and Kwack, 1963). If the calcium ion concentration is too low or too high, the physiology of the pollen tube cytoskeleton was affected, causing the upper part of the pollen tube to form a thick wall, thereby performing the pollen tube to shrink, become thinner, deform and become fragile (Wasag *et al*, 2022). This study used single-factor to examine the effect of different Ca^{2+} levels on the pollen germination and pollen tube growth of Caoyuan No.1 and Zhilixing. According to the obtained results (Fig 1E-F), In basal media with a calcium ion concentration of 100 mg/L, Caoyuan No.1 has the highest germination rate of 65.33% and the longest pollen tube of 154.40ìm. The longest pollen tube length of Zhilixing occurred at a concentration 150 mg/L and the highest germination rate was recorded at a concentration of 100 mg/L. Different plants and species may need different calcium ions concentration to support their pollen development (Liu *et al*., 2013). At 150 mg/L to 200 mg/L, pollen tube length and pollen germination rate gradually decreased in linearly with increasing calcium ions concentration.

Table 2: Orthogonal experiment design (three factors and four levels).

Level	Factor		
	Sucrose %	Boric acid ($mg \cdot L^{-1}$)	Calcium chloride ($mg \cdot L^{-1}$)
1	2	100	50
2	5	150	100
3	10	200	150
4	15	300	200

Selection of the optimal culture medium

The most suitable medium for in vitro pollen germination varies by species, or even by different flowering states to collect pollen for the same species. Hemerocallis (Qiu *et al*., 2021) has the highest percentage of *in vitro* germination occurred with the concentrations of 4 g L agar, 74.6 g L sucrose, 800 mg L boric acid and 590 mg L calcium nitrate; Passiflora spp. Orthogonal experimental design (OED) is a design method for the study of multi-factor and multi-level systems and can find the optimal level combination through a small number of experiments. Therefore, the optimum medium for pollen viability of most species has been determined using OED, which can save much manpower and material resources compared with comprehensive experiments (Jiuru and Zhihui, 1995; Chengyi and Jie, 2004). This study also used OED to screen the optimum medium for in vitro germination of alfalfa and *Medicago ruthenica* pollen. Table 3 shows that the highest pollen germination of Caoyuan No.1 occurred in T12 (78.56%), followed by T10 (71.33%) and T11(68.11%). The pollen germination rate of Zhilixing treated with T11 (70.79%) was the highest. The R values for both were in the order: RA >RB >RC (Table 4, 5). These results indicate that the concentration of sucrose serves essential functions in pollen germination and tube length, followed by the concentration of H_3BO_3, followed by the concentration of $CaCl_2$. The results from this study agreed with those obtained by Fagundes, *et al.* (2021) who noted a huge impact of the results of *in vivo*

Table 3: Pollen germination rate and pollen tube growth in orthogonal experiments.

Treatment	Sucrose	Boric acid	Calcium chloride	CY		ZB	
	%	($mg \cdot L^{-1}$)	($mg \cdot L^{-1}$)	PGR/%	PTL/μm	PGR/%	PTL/μm
1	2	100	50	34.54hi	97.52h	29.70h	84.01f
2	2	150	100	40.121hg	116.55efg	33.18hg	104.27e
3	2	200	150	33.31i	122.73def	35.10gfh	90.63f
4	2	300	200	33.81i	105.37gh	35.21gfh	104.10e
5	5	100	100	51.12de	139.63ab	47.83de	110.64de
6	5	150	150	56.10cd	125.82cde	53.72cd	115.37dce
7	5	200	200	58.91c	121.278ef	56.42bc	116.96bcde
8	5	300	50	42.34fg	110.08fgh	39.18fg	104.93e
9	10	100	150	60.55c	148.97a	60.27b	111.05de
10	10	150	200	71.33b	143.55ab	50.78cde	127.51abc
11	10	200	50	68.11b	135.12bcd	70.79a	135.79a
12	10	300	100	78.56a	150.53a	53.14cd	135.73a
13	15	100	200	48.46ef	119.64ef	46.99e	123.42abcd
14	15	150	50	52.30de	137.58abc	39.61f	129.42ab
15	15	200	100	45.97efg	111.41fg	34.46gfh	114.20de
16	15	300	150	43.93fg	104.79gh	40.04f	108.16e

pollen germination of Hylocereus with the composition and concentration of the medium. However, there are also studies that are contrary to our and the abovementioned results. In studies conducted by Mondo *et al.* (2021), the pollen genotype and the growing conditions, such as the incubation temperature, the medium viscosity and time to use became the main actors that affect pollen germination in individual species. However, the medium composition appears to have little effect on pollen germination. The pollen of Caoyuan No.1 had the best germination rates and the growth of the pollen tube on the media contained a sucrose concentration of 10% and a concentration of boric acid of 300 mon/L, a concentration of calcium ions 100 mon/L. A sucrose concentration of 10%, a concentration of boric acid of 200 mon/L, a concentration of calcium ions 50 mon/L is regarded as the optimal feature combination of Zhilixing. The above results were consistent with previously reported research by authors on other types of alfalfa. (Lehman, 1964) No effect of incubation time and temperature on pollen germination and tube growth, has been previously reported for alfalfa (Brunet, 2019). However, other factors such as the effect of sampling date, hormones and clump formation on the germination and growth of alfalfa pollen need further exploration.

Comparison of pollen viability of different species

The method for *in vitro* pollen germination was used to compare the pollen viability of different varieties using the best medium. Boxplot diagrams were used to display the differences visually between varieties. The pollen germinability of the four cultivars was good by microscopic observation, ranging from 34.61% to 59.76%. Caoyuan No.1 showed the highest vitality value, meaning it was more suitable as the male parent for hybrid breeding

Table 4: Intuitive analysis of orthogonal experiment (CY).

Index	PGR/%			PTL/μm		
	Sucrose %	Boric acid (mg·L^{-1})	Calcium chloride (mg·L^{-1})	Sucrose %	Boric acid (mg·L^{-1})	Calcium chloride (mg·L^{-1})
K_1	141.78	194.66	197.282	442.178	505.76	480.3
K_2	208.47	219.846	215.77	496.808	523.508	518.128
K_3	278.54	206.304	193.898	578.17	490.538	502.31
K_4	142.2	198.64	212.506	473.42	470.77	489.838
R	34.19	22.14	6.29	34	33.99	13.19

Note: K_i was obtained by summing the total number of columns corresponding to level. R represents the range.

Table 5: Intuitive analysis of orthogonal experiment (ZB).

Index	PGR/%			PTL/μm		
	Sucrose %	Boric acid (mg·L^{-1})	Calcium chloride (mg·L^{-1})	Sucrose %	Boric acid (mg·L^{-1})	Calcium chloride (mg·L^{-1})
K_1	133.19	184.79	179.28	383.01	383.012	454.15
K_2	197.15	177.29	168.61	447.9	476.57	464.84
K_3	234.98	196.77	189.13	510.08	457.58	425.21
K_4	161.1	165.57	189.4	475.2	452.92	471.99
R	25.447	18.49	7.3	31.77	31.767	11.86

Note: K_i was obtained by summing the total number of columns corresponding to level. R represents the range.

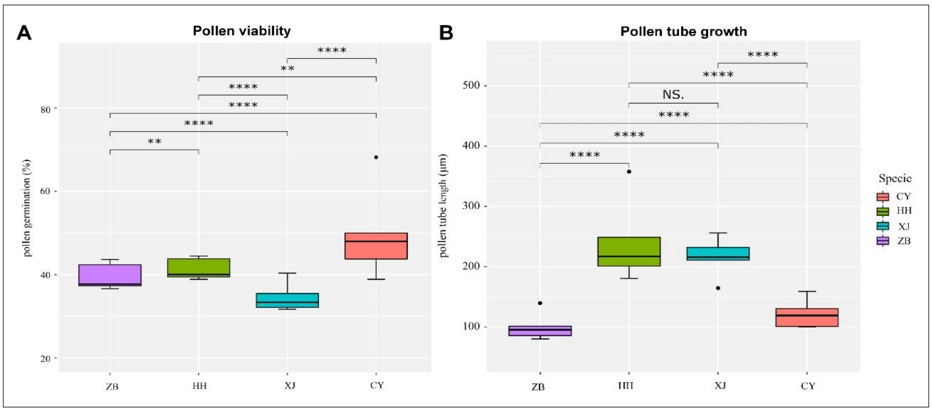


Fig 2: Comparison of pollen viability (A) and pollen tube growth (B) of different species.

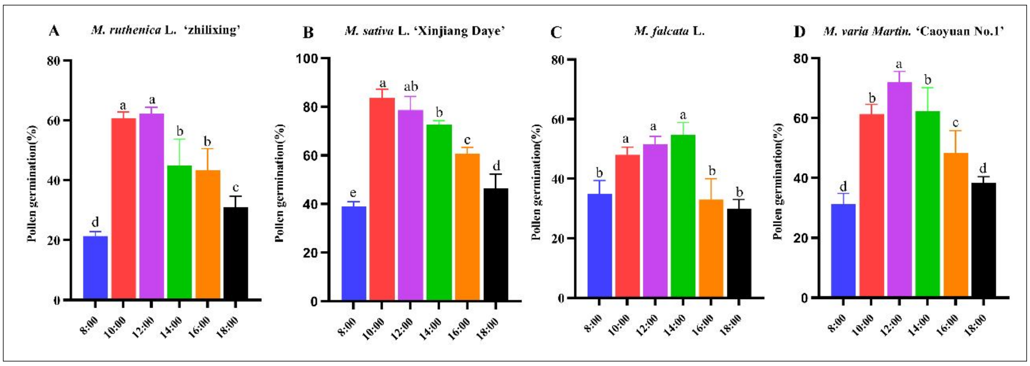


Fig 3: Diurnal variation of pollen viability of different species.

(Fig 2A). The grown cultivar could be shown to have a significantly higher pollen germination rate than the feral population. This is consistent with findings on oilseed rape (Lankinen *et al*, 2018). There was a significant difference in pollen tube length between four species(Fig 2B). Pollen germination rate was significantly influenced by plant category. The wild-type Medicago falcata in Xinjiang has longest pollen tube. Our results show that there was no relationship between pollen viability and pollen growth. Fig 3 shows dynamic changes of pollen viability between different species. The highest pollen germination of *M. ruthenica* L. Sojak 'zhilixing' (Fig 3A) and *M. varia* Martin. 'Caoyuan No.1' (Fig 3D) appeared at 12:00 p.m. The highest pollen germination of *M. sativa* L. 'Xinjiang Daye' (Fig 3B) and *M. falcata* L. (Fig 3C) appeared at 10:00 a.m. and 14:00 p.m. The daily pollen activity curves of the four varieties showed a single peak type, reaching the highest point around 12:00 p.m. and the pollen germination rate began to decline after 14 p.m.

CONCLUSION

Pollen grains were used in an in vitro culture experiment to show that sucrose, boron and calcium can promote pollen germination and pollen tube growth of *M. ruthenica* L. Sojak 'zhilixing' and *M. varia* Martin. 'Caoyuan No.1 within a certain concentration range. The results show that the best solid medium for pollen germination of *M. ruthenica* L. Sojak 'zhilixing'was a medium containing 10% sucrose, 300 mon/L H_3BO_3 and 100 mon/L $CaCl_2$; that of *M. ruthenica* L. Trautv. 'zhilixing' was 10% sucrose, 200 mon/L H_3BO_3 and 50 mon/L $CaCl_2$. This finding serves be used to choose male materials with high pollen vigor for cross-pollination, which can directly affect the seed-setting rate of hybrid combinations.

ACKNOWLEDGEMENT

FL S designed the experiment, RR S performed the experiments and wrote the manuscript. Thanks to Key Laboratory of Grassland Resources, Ministry of Education P.R. of China. This study was supported by the the National Natural Science Foundation of China (32160323), Key Projects in Science and Technology of Inner Mongolia (2021ZD0031) and Major demonstration project of "the open competition" for seed Industry science and technology innovation in Inner Mongolia (2022JBGS0016). The authors would like to express their gratitude to Edit Springs for the expert linguistic services provided.

Conflict of interest: None.

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