

**Pollen Dispersion, Pollen Viability and Pistil Receptivity in *Leymus chinensis***

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- **Background and Aims** *Leymus chinensis* is an economically and ecologically important grass that is widely distributed across eastern areas of the Eurasian steppe. A major problem facing its propagation by man is its low sexual reproductivity. The causes of low fecundity are uncertain, largely because many aspects of the reproductive biology of this species remained unknown or incomplete. This study aims to address some of these issues.

- **Methods** Pollen dispersion, pollen viability, pollen longevity and pistil receptivity were studied in a representative, natural population of *L. chinensis* growing in Inner Mongolia.

- **Key Results** Flowering of *L. chinensis* occurred at the end of June and lasted for 5 d. Pollination peaked between 1600 h and 1700 h, and about 56.1 % of the total pollen grains were released at this time. Pollen density was highest towards the middle of flowering spikes and lowest at the bottom over the 5 d measurement period. Pollen viability (62.4 %) assessed using TTC was more accurate than using IKI (85.6 %); 50 % of pollen arriving on stigmas germinated. Pollen remained viable for only 3 h and the pollen : ovule ratio was 79.3 : 1. Pistil receptivity lasted for only 3 h and, overall, 86.7 % of pistils were pollinated. Within the spike, the relative fecundity of different positions was middle > lower > upper throughout the period of pollination; daily variation of fecundity was similar to that of the pollen flow. The spikes that opened on the day of highest pollen density exhibited the highest fecundity (36.0 %). No seeds were produced by self-pollination.

- **Conclusions** The data suggest that low pollen viability, short pollen longevity and short pistil receptivity all appear to contribute to the low seed production typical of this important forage crop.

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**Key words:** *Leymus chinensis*, pollen dispersion, pollen viability, pistil receptivity, fecundity, laser scanning confocal microscopy.

**INTRODUCTION**

*Leymus chinensis* (*Aneurolepidium chinensis*), a member of the family Gramineae, is a perennial rhizome grass (Kuo, 1987). Due to its excellent stress tolerance (Koyama, 1987; Yin et al., 1993; Xiao et al., 1995), grasslands dominated by *L. chinensis* are widely distributed at the eastern end of the Eurasian steppe, from North Korea westward to Mongolia and northern China, and north-westward to Siberia. Early spring emergence and rapid growth, high palatability and herbage production make the grasslands ideal for grazing and forage production (Li et al., 1983). Furthermore, because of its high vegetative propagation and abundant horizontally creeping rhizomes, *L. chinensis* is used as a soil-binding plant to protect soil from desertification in the arid areas of northern China.

Because *L. chinensis* is an economically and ecologically important grass, the species has received considerable attention (Wang, 1984). However, a major problem facing its propagation is its low sexual reproductivity. To better understand the causes of the species’ low fecundity, several investigations have been conducted (Wang, 1984, 1998; Huang et al., 2002); however, these have focused on the effects of ecological factors. Few investigations (Ma et al., 1984; Zhao and Tu, 1993) have dealt with its sexual reproduction. It has been reported that seed production under natural conditions can be <40 % (Wang, 1984). Seed production by *L. chinensis* may be influenced by climate (Guo and Zhu, 1994; Gao et al., 1999; Yang et al., 2000), nutrient uptake (Yang, 1989; Wang, 1998) and vegetative growth (Wang, 1998, Wang and Ripley, 2000), as well as by disturbance by men and animals (Yang and Zhu, 1988; Yang and Zhu, 1989; Wang, 2000). However, the causes of low fecundity are uncertain, largely because many aspects of the reproductive biology of this species remained unknown or incomplete. The objectives of this investigation therefore were to measure the pattern of pollen dispersion, pollen viability and pistil receptivity during the pollination period under natural conditions, and to evaluate the effects of these factors on seed production.

**MATERIALS AND METHODS**

**Site description**

The study site was located south of the Xilin River basin, Inner Mongolia, China (43°32′58″N, 116°40′34″E), approx. 1265 m a.s.l. It has a temperate, semi-arid climate with a mean annual rainfall and temperature of 350 mm and 0-3 °C, respectively (Chen, 1988). The permanent plot of *L. chinensis* belongs to the Inner Mongolian Grassland Ecosystem Research Station and it has been free from grazing since it was fenced in 1979.
Pollen dispersion

Pollen traps constructed from petroleum jelly on microscope slides were attached to vertical wooden laths facing the direction of the prevailing wind (Mulugeta et al., 1994). Laths were driven into the ground, with the slides at heights of 0.38, 0.45 and 0.52 m above the soil surface, corresponding to the lower, middle and upper positions of the spikes, respectively (two replicates for each position). Pollen was collected daily from 21 to 31 June 2002. From 21 to 25 June, the slides were set at 0800 h and changed at 1800 h. From 26 to 31 June, the slides were set at 0800 h and changed at 1200 h, 1400 h, and then at every hour till 1800 h. The slides set at 1800 h were collected at 0800 h the following day. During the collection periods temperature and relative humidity were recorded. The highest temperature was found between 1400 and 1500 h, and then it gradually declined. Relative humidity was highest between 1400 and 1600 h and declined to a minimum between 1600 and 1700 h, after which it increased again.

Pollen density counts were taken from five random fields of view (field size = 20 mm$^2$) per slide under a microscope (Olympus BH-2) at ×40 magnification without staining. *Leymus chinensis* pollen could be identified and distinguished from that of other species by its shape (spherical) and size (30–7±35–2 μm) (Wan and Wei, 1999). The data collected were used to estimate the temporal and spatial distribution of pollen flow.

Pollen viability and longevity

*Leymus chinensis* began to flower on 26 June, and produced pollen for about 5 d. During the pollination days, fresh pollen was collected daily at 1500 h by placing the anthers in Petri dishes just before anthesis. Pollen was stained with either 1,2,3-triphenyl tetrazolium chloride (TTC) (1.0 % by weight in 50 % sucrose) or iodine–potassium iodide (IKI) (Mulugeta et al., 1994). Freshly harvested pollen was dusted onto a microscope slide with a brush to which four or five drops of stain were added. Then the slide was immediately covered with a coverslip and the edges sealed with nail varnish. Pollen was observed 10 min after staining with IKI, while pollen grains stained with TTC were examined after 15–30 min incubation at 40 °C. The percentage of pollen out of 300–500 grains per slide (three replicates for each staining treatment) that exhibited the appropriate staining reaction was determined using an Olympus Vanox microscope at ×100 magnification. Control experiments were performed using heat-killed pollen (80 °C for 2 h; Dafni and Firmage, 2000).

Pollen longevity was measured at room temperature. Twenty fresh anthers were selected from ten plants at random; ten were kept on dry blotting paper in Petri dishes and the other ten were kept in Petri dishes containing water-saturated blotting paper. The viability was then tested by TTC at 0, 1, 2 and 3 h after the anthers dehisced. Pollen longevity was estimated by counting the number of stained pollen grains out of 300–500 pollen grains per slide.

Pistil receptivity, pollination success and pollen tube growth in vivo

To determine the quantity of pollen grains on the pistil and in vivo pollen tube growth, the spikes from which pistils were collected were tagged at six different times (0, 1, 2, 3, 24 and 27 h) from flower opening. After fixation in 2.5 % glutaraldehyde, the pistils were kept in 70 % ethanol, and then transferred to 10 % aqueous sodium sulfite, autoclaved at 160 kPa and 120 °C for 15 min, and stained in decolourised aniline blue (aniline blue WS 0·1 % in aqueous 0·1 M K$_3$PO$_4$, 10 % (w/v) glycerol). Pistils were then squashed in stain and sealed with petroleum jelly to prevent dehydration (Williams et al., 1982). All preparations (30 pistils for each time period) were examined using a laser scanning confocal microscope (Bio-Rad 1000 laser scanning confocal microscope) with the filter combinations of KP490, KP500, RH 510 and LP 528. The pollination success was calculated as the percentage of pollinated pistils after open pollination (Tangmitcharoen and Owens, 1997).

Pollen production and seed set

To study floral morphology, 30 mature flowers were collected and observed using a dissecting microscope (Opton 47 52 00-9901). Samples for light microscopy were fixed in FAA (formalin–acetic acid–alcohol) and were then sectioned (6–8 μm) after being embedded in paraffin wax (Jensen, 1962). To determine the number of pollen grains per flower, one mature anther per flower was removed from five unopened flowers of five different spikes. The anthers were gently squashed in 1 ml of TTC, the pollen grains transferred to a graticule slide and counted using an Olympus BH-2 microscope at ×100 magnification. The pollen : ovule ratio was determined by dividing the average number of pollen grains per flower by the average number of ovules per flower. During the anthesis period, ten spikes were tagged every day. The numbers of seeds set were counted 2 months after pollination. Fecundity was calculated by dividing the number of seeds set 2 months after pollination by the total number of flowers at pollination from the ten spikes tagged.

Data analysis

Means (± standard error) were calculated for all the measurements using Excel (2000) or SPSS vs10.0 software for Windows (10-0). Analysis of variance (ANOVA) was used to assess the variation of the data. Duncan’s new multiple range test at $P < 0.05$ was used to compare the means and determine the significance of differences between variables.

RESULTS

Floral morphology

*Leymus chinensis* flowers are hermaphroditic and arranged in compound spikes. There were approx. 45 ± 5 spikes m$^{-2}$, and the plant height ranged from 0.4 to 0.6 m with 0.14 ± 0.02 mm spikes at the time of flowering. At the base of each
spikelet, there is a pair of sterile glumes that surrounds a series of flowers (five to eight in number). Each flower has a lemma (outer bract) and a palea (inner bract) at its base, and contains two lodicules and one pistil surrounded by three anthers. The mature pistil has two laterally feathery stigmas and a hairy ovary (Fig. 1A). Each anther has four microsporangia arranged in pairs in the two lobes (Fig. 1B). The ovary has one locule, containing one anatropous ovule. The ovary is superiorly positioned with basal placentation (Fig. 1C). The flowers usually open synchronously or basipetally within a spike between 1400 h and 1800 h. Some of the upper flowers begin to open at 1400 h. At 1500 h, several middle flowers begin to open. Most of the middle flowers and lower flowers opened synchronously between 1600 h and 1700 h, while a few flowers open after 1700 h.

**Pollen dispersion**

In this study, few plants flowered before 26 June, and no pollen grains were collected from 21 to 25 June. Pollen dispersal began on 26 June. The daily pollen density increased to a peak on 28 June, and then decreased (Fig. 2). After 30 June, <5 % of the spikes in this field produced pollen.

Variability was found in pollen density between different periods of collection within an individual day and between the different positions within a spike, as shown in Fig. 3. The variation in pollen density during each day followed a similar trend at the three different positions within a spike. Pollen dispersal began at 1400 h, but at low density until 1600 h. The peak occurred between 1600 and 1700 h, during which period 56 % of the total pollen liberated that day was collected over the entire spike (Fig. 3). After 1700 h, pollen density sharply decreased to 55-54 × 10^4 grains m⁻².

Variation was also observed in pollen density between different positions in a spike. For each collection period, the number of pollen grains was highest in the middle portion of the spike and lowest in the upper portion of the spike. The highest pollen density occurred between 1600 and 1700 h at the middle position (330-63 × 10^4 grains m⁻²). The lowest pollen density (44-50 × 10^4 grains m⁻²) occurred at the lower position between 1700 and 1800 h. The highest mean of pollen density (148-0 × 10^4 grains m⁻²) was found in the middle position and the lowest (109-3 × 10^4 grains m⁻²) was found in the lower position; that of the upper position was 128-5 × 10^4 grains m⁻².

**Pollen viability and longevity**

Different staining methods gave different assessments of pollen viability. Viability was 62 ± 5 % when stained with TTC (Fig. 1D), but was 86 ± 3 % when stained with IKI (Fig. 1E). The difference between the two treatments was highly significant (P < 0-05). After fresh pollen was heat-killed for 2 h at 80 °C, no colour reaction was observed with TTC (Fig. 1F). However, most of the heat-killed pollen treated with IKI stained in the same manner as fresh pollen (Fig. 1G).

Staining with TTC showed that pollen can survive for about 3 h at room temperature. Pollen viability was about 71 % at the moment of anther dehiscence. When pollen was kept at room temperature in Petri dishes, viability decreased quickly. After 1 h, the viability declined to 56 % in wet conditions and to 44 % in dry conditions; after 2 h it decreased further to 34 % and to 18 %, respectively. Although the viability of the pollen kept in wet conditions decreased more slowly than in dry conditions, under both conditions viability decreased to below 5 % after 3 h (Fig. 4).

Pollination began when pollen grains landed on the surface of the stigma, which had the appearance of a meadow of finger-like papillary cells. The pollen germinated in about 3 h, and pollen tubes emerged and grew down the style. The tubes were readily visualized by staining callose with aniline blue (Fig. 1H and I). It was found that approx. 50 % of the pollen germinated under the conditions of the experiment.

**Pistil receptivity**

Pistil receptivity lasted for about 3 h after anthesis. Laser scanning confocal microscopy showed that, pre-anthesis, almost all the stigmatic surface was small and smooth without pollen grains. As the pistil emerged from the bracts, the style elongated and the stigma expanded markedly in size and, finally, became receptive to pollen. The number of pollen grains reaching the stigma (pollination success) gradually increased during the first 3 h of the period of receptivity and then reached a plateau. About 67-7 % of pistils were pollinated at the time of anthesis (1500 h); however, on average, there was less than one pollen grain per pistil at this time. There was a significant increase in pollination between 1500 and 1700 h. At 1800 h approx. 86-7 % of pistils were pollinated with an average of 16-5 pollen grains per pistil. There were no further significant increases thereafter (Figs 5 and 6). Thus, the most effective pollination period was between 1500 and 1800 h in terms of both pistils pollinated and the number of pollen grains per pistils (Figs 5 and 6).

**Pollen production and fecundity**

There was not much difference in the number of pollen grains produced per anther between spikes (26 444 ± 1799, n = 10). The pollen : ovule ratio was 79 333 : 1. Variation was observed in fecundity in relation to different densities of pollen flow. Within an individual spike, fecundity was 24-3, 35-5 and 30-8 % for the lowest, middle and upper positions of the spike, respectively (Fig. 7), and correlated with the relative pollen densities at these positions.

Over the whole period of pollination (26-30 June), the variation in fecundity tended to be similar to that of pollen flow (Fig. 2). Fecundity varied as the spikes opened from day to day, and the mean value was 30-2 % for all the spikes. It increased from 26 to 28 June, followed by a decline thereafter. After 30 June, fecundity dropped to 16-5 %. No seeds were produced if spikes were covered with bags.
DISCUSSION

It is common for hermaphroditic angiosperms to produce more flowers and ovules than fruits and seeds (Willson, 1979; Bawa and Webb, 1984; Sutherland and Delph, 1984; Sutherland, 1986). To explain the existence of these non-fruited flowers, two hypotheses have been put forward (Sutherland, 1987). One proposes that resources other than pollen limit seed production. The other hypothesis proposes that seed plants typically do not receive enough pollen for full fruit- or seed-set. The amount of pollen produced by a flower reflects the probability that a sufficient number of...
pollen grains will reach a stigma (Cruden, 1977). In the present investigation, pollen production was prolific as shown by the high pollen : ovule ratio (79 333 : 1). According to the definitions proposed by Cruden (1977), L. chinensis should be classified as exhibiting obligate xenogamy (the highest outcrossing level). In addition, the lack of seed production in the spikes covered with bags also supports the view that L. chinensis is normally outcrossing and possesses strong barriers to self-fertilization.

In several xenial species, it has been reported that the pistils do not receive enough pollen for fruit- or seed-set and that the fecundity can be raised by supplemental hand pollination (Bawa and Webb, 1984; Zimmerman and Pyke, 1988; Johnston, 1991). Ma (1984) observed a low density of pollen flow due to asynchronous dehiscence in a cultivated population of L. chinensis, resulting in low fecundity. However, in the current study it was found that dehiscence in the natural population was generally synchronous in terms of pollen dispersion. Over 95 % of the spikes flowered within 5 d between 26 and 30 June, and 56-15 % of the total pollen was concentrated between 1600 and 1700 h in the 4-h collection period. Correspondingly, the highest fecundity occurred on 28 June, while the lowest fecundity was found in the spikes that flowered after 30 June. The similar trend of variation in both seed-setting and pollen density indicated that a high fecundity was consistent with a high pollen density on the day of flowering. It was further observed that variation in fecundity correlated positively with pollen density within an individual spike, as both the highest fecundity and highest density of pollen flow appeared halfway up the spikes (Fig. 7). From this positive correlation, it is deduced that fruit- and seed-set can be limited by pollen load, particularly in the lower spikes and beyond the period 26–30 June. Even in the upper spikes and within the period 26–30 June, the frequency of successful pollination can be lowered when and/or where pollinators are unreliable, since xenogamous species are primarily outcrossers or self-incompatible (Cruden, 1977).

Pollen viability is considered as an important parameter of pollen quality (Dafni and Firmage, 2000). Staining with IKI and TTC are common techniques used to determine pollen viability (Mulugeta et al., 1994; Shirazi and Muir, 1998; Zhou et al., 1999; Dafni and Firmage, 2000). Ma et al. (1984) reported that pollen viability was about 90 % when tested with IKI, while the fecundity was below 50 %. However, in the study presented here, the pollen viability was 85.6 % and 62.4 % after staining with IKI and TTC.
respectively. The difference between these two treatments was highly significant \( P < 0.05 \), demonstrating that it is essential to correlate staining techniques with \textit{in vivo} methods before trusting either of the pollen staining reactions as an indicator of viability (Fritz and Lukaszewski, 1989; Sedgley and Harbard, 1993). In this study, it was found that IKI staining indicated an unrealistically high viability, whilst the TTC reaction produced a reasonable indication of viability when compared with the germination rate of pollen grains \textit{in vivo}. Furthermore, this discrepancy was reflected in the colour reaction of heat-killed pollen. IKI staining clearly failed to distinguish fresh from heat-killed pollen, whilst TTC staining easily separated the two.

Pollen longevity is another important factor related to fecundity that might limit seed production (Fritz and Lukaszewski, 1989; Dafni and Firmage, 2000). The experiments described here indicated that pollen viability can decline sharply from 70-8 \% to <5 \% after 3 h. In other words, pollen of this species is short-lived and pollination may therefore fail if the pollen reaches the pistil after a delay of longer than 3 h. Since \textit{L. chinensis} is an anemophilous species, low pollen viability could be associated with the high temperatures and low relative humidity typical of this arid area.

In many angiosperm species, pistil receptivity can last for one or several days. A long duration of pistil receptivity helps high pollination success (Nepi and Pacini, 1993; Tangmitcharoen and Owens, 1997; Sornsathapornkul and Owens, 1998; Aleemullah \textit{et al}. 2000). The results presented here indicate that pistil receptivity of \textit{L. chinensis} lasted for only about 3 h. Beyond this time, pistils will usually fail to be pollinated no matter how high the viability and the density of pollen. There was a seeming conflict between the higher pollen density and the lower fecundity at the upper position of the spike (Fig. 7). As the flowers at the upper position frequently opened earlier in the day (1400 h) than those in the other positions (1600–1700 h), pollen density was low at that time. Indeed, pollen density increased (1600–1700 h) when the pistil receptivity would have been significantly decreasing. It would appear that the short pistil receptivity is an adverse factor for seed production in this species.

In summary, the present work has revealed details of pollen dispersal, pollen longevity and pistil receptivity in a natural population of \textit{Leymus chinensis}. In particular, it has shown that variation in pollen density and fecundity occurs
between different positions within individual spikes and also between spikes. In addition, pollen viability, pollen longevity and pistil receptivity were found to be closely related to fecundity levels. The data presented here suggest that these factors would limit the overall amount of seed production by *L. chinensis* under natural conditions. Further study of megalosporogenesis, fertilization and embryogenesis in this species would help towards understanding the causes of its low fecundity.

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LITERATURE CITED


