Australian Journal of Crop Science AJCS
AJCS 5(12):1603-1609 (2011) ISSN:1835-2707

Screening for natural male sterile mutants in alfalfa (*Medicago sativa* L.) varieties

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Abstract

Although male sterility is important in obtaining heterosis, there is no widespread exploitation of male sterility in alfalfa breeding. Male sterile mutants could provide the basic materials for carrying out seed production in breeding programmes. In order to establish rapid screening methods from which it is feasible to obtain natural male sterile mutants in alfalfa, methods of pollen production and pollen viability were compared by analysing the genetic variance in 19 alfalfa varieties. Both pollen production and pollen viability varied significantly among varieties, and much higher variations existed within varieties than between varieties. It was determined that there was 87.87% variability within varieties for pollen production and 90.85% for pollen viability. No significant difference was observed between different reproductive branches (P=0.589). Screening by individual plant method, there were 58.48% plants with high pollen production, 33.33% with moderate pollen production, 7.36% with trace pollen production, and 0.83% with no pollen production. In total, 6 male sterile mutants were identified from varieties Graze401+z (*ms1, M-fon1* and *M-fon2*), Longzhong (*ms2*) and Big west (*ms3* and *ms4*), in which four mutants (*ms1-ms4*) were male sterile but female fertile mutants and two were multi-pistil male sterile mutants (*M-fon1* and *M-fon2*). The mutants *ms1-ms4* showed male sterility accompanied with abnormal anther development and pollen grains degeneration. *M-fon1* and *M-fon2* mutants developed flowers with multi-pistil, as well as no pollen, thereby displaying both male and female sterility.

Keywords: male sterile mutant; *Medicago sativa* L.; pistil; pollen production; pollen viability.
Abbreviations: PP-pollen production; PV-pollen viability; SEM-scanning electron microscope; TTC-2, 3, 5-Triphenyl tetrazolium chloride.

Introduction

Alfalfa (*Medicago sativa* L.) is one of the most important leguminous forage crops. It is a hermaphroditic flower and is monothelious, as well as being a typical perennial autotetraploid and allogamous plant (Riday and Brummer, 2002; Wang et al., 2011c). To date, more attention has been paid to other factors, such as the improvement of yield (Li and Brummer, 2009) or biotic and abiotic tolerances (Musial et al., 2005; Narasimhamoorthy et al., 2007; Wang et al., 2011b), but has been less focused on seed production (Rosellini, 2004). Seed yield of alfalfa is important in determining the effective distribution of new cultivars to farmers (Bolaños-Aguilar et al., 2002). It is interesting that although alfalfa has a large potential for phytomass production and has large numbers of flowers present, seed set is generally low (Iannucci et al., 2002; Wang et al., 2011a). Thus, a major research effort should be directed at exploiting and manipulating reproductive mechanisms in forage legumes, especially to increase efficiency by making the actual seed yield close to the biological seed production potential (Falcinelli, 1999). Most commercial varieties of alfalfa are synthetics and an undesirable feature of these synthetic varieties is a decline in yield with generations. On the other hand, the genetic constitution of alfalfa synthetics could change due to inbreeding effect, natural selection, non-random mating and other factors (Li and Brummer, 2009). Therefore, a key problem in improving yield in alfalfa breeding is that of utilizing heterosis and producing hybrid seeds economically (Brummer, 1999; Riday and Brummer, 2002; Robins et al., 2007). Male sterility could provide the basic materials for carrying out seed production improvement. Male sterility systems are well characterized and utilized for hybrid seed production in some crops such as rice (Li et al., 2011), wheat (Adugna et al., 2004) and maize (Palmer et al., 2011). In the 50-80s of 20th century, Davis and Greenblatt (1967), Bradner and Childers (1968), and Pedersen and Stucker (1969) have reported several natural male sterile mutants in alfalfa breeding programmes. However, to date there is no widespread exploitation of male sterility in alfalfa breeding, although male sterility is important in heterosis utilization (Suginobu, 1976). It has been reported that hybrid alfalfa cultivars may become economically competitive if higher levels of heterosis are obtained in this crop, so the availability of dependable sources of male sterility may become very important in this perspective (Rosellini et al., 2001). The overall goal of this research was to establish a rapid protocol to screen and find natural male sterile mutants in alfalfa. The ultrastructure of anthers and pistils in the male sterile mutants were also studied.

Results

Production and viability of alfalfa pollen in different varieties

Pollen production of the 19 alfalfa varieties showed either

moderate amounts of pollen (class 3) or large amounts of pollen (class 4) dehisced when the flower was tripped (Fig. 1). There was a significant difference in alfalfa pollen production within and among the varieties ($P < 0.001$), which accounted for 87.87% of the difference within varieties but only 12.13% between varieties, and there was no significant difference of pollen production between different reproductive branches (Table 2). Since there is high genetic variation within each variety, the individual plant scoring method found that there were 58.48% plants with high pollen production (class 4), 33.33% with a moderate amount of pollen production (class 3), 7.36% with trace pollen production (class 2) and 0.83% with no pollen production (class 1) (Fig. 2). Pollen viability showed wide variations between the 19 alfalfa varieties in this study (Fig. 1). Compared to pollen production, a higher variation of pollen variability existed at 90.85% within varieties while it was 9.15% between varieties (Table 2). The rate of pollen abortion of individual plants ranged from 0.53% to 73.14%.

Natural male-sterile mutants screened in alfalfa varieties

In total, 16 male-sterile plants were found in these 19 alfalfa varieties using the pollen production method in 2009. The pollen production and pollen viability of these male-sterile plants was tested again in 2010 and 2011 to ensure the robustness of these results. Our results showed that six out of 16 male sterile plants were stable for male sterile traits. These spontaneous mutants were designated as ms*1*, *ms2*, *ms3* and *ms4*, while two multi-pistil mutants designated as *M-fon1* and *M-fon2,* and six male sterile mutants were identified from varieties Graze401+z (*ms1, M-fon1* and *M-fon2*), Longzhong (*ms2*) and Big west (*ms3* and *ms4*). The male-sterile mutants showed shriveled anthers with no pollen grains (Fig. 3, A) compared to the normal plant which expressed healthy anthers with a large quantity of pollen grains (Fig. 3, B). Furthermore, the male-sterile mutants showed shriveled anthers without cracking (Fig. 3, C), compared to the normal plants which expressed anther dehiscence with numerous pollen grains (Fig. 3, D). The multi-pistil mutants had two or three pistils in the floret (Fig. 3, E and F) and the phenomenon of multi-pistil emerged at an early stage of the floret development.

Ultra structures of male sterile mutants

The ultra-structures of stamen and pistil in these six male sterile mutants were observed under scanning electron microscope (SEM). The analysis of *ms1* showed that pollen grains had irregular shapes, where the surface of the pollen grains began to shrink from the germinal furrow, then in different planes, and lastly the contraction surface disappeared (Fig. 4, A). The anther of *ms2* shriveled and cracked in the late stages of development and no pollen grains existed (Fig. 4, B). Another mutant *ms3* was belonging to anther un-dehiscence type, which had normal anthers full of pollen grains but was defective in anther dehiscence during the tripping process (Fig. 4, C). The mutant *ms4* had short floral bud and small anthers in the late developing stages (Fig. 4, D). The flowers of *M-fon1* and *M-fon2* mutants developed with multi-pistil as well as no pollen. These flowers are, therefore, both male sterile and female sterile. Scanning electron microscope observations showed that the stigma and ovule number in the ovaries of *M-fon1* and *M-fon2* were lower than those of normal plants. The pistils of *M-fon1* and *M-fon2* also showed variations in ovary size and contained fewer ovules in the ovary, or even no ovary ovule in the small pistil (Fig. 4, E and F). This suggests a high degree of degradation in female sterility.

Discussion

Is there efficient proposal for screening natural male sterile mutants in alfalfa?

For the development of hybrid alfalfa, the use of male sterility is the most efficient method to control pollination (Okumura et al., 1995). The first natural male sterile mutant in alfalfa was reported by Childers (1952) who found a recessive genetic (nuclear) male sterility system characterized by complete degeneration of microspores. The genetic-cytoplasmic (nuclear-cytoplasmic) sterility system showed varying degrees of male sterility, was reported by Davis and Greenblatt (1967). Alfalfa male sterile plants were also obtained by genetic transformation with a construct containing the *Barnase* gene under the control of a tobacco anther tapetum specific promoter (Rosellini et al., 2001). The male sterile mutants reported above made it possible to use heterosis in an alfalfa breeding programme, but little was known about the screening efficiency for spontaneous male sterile mutant in alfalfa. It has been reported that the search for natural male-sterile plants from more than 50,000 field-tested plants resulted in the selection of one fully sterile and nine partially sterile plants, and cytological observations of all 10 plants revealed empty, shrunken pollen coats in shrunken anthers (Bradner and Childers, 1968). According to the technique described by Childers and McLennan (1960), plants having flowers with no pollen or with pollen in highly reduced amounts were screened in 19 varieties in this study. Considering the number of plants tested and the genetic variability of the tested materials, we reached the conclusion that a 0.83% chance of spontaneously finding alfalfa plants with no pollen grains using this pollen production method was efficient. It has been previously reported that the amount of dehisced pollen in alfalfa plants is not significantly affected by flower position on the plant, sampling dates, or interactions between clones and positions and between positions and sampling dates (Barnes and Garboucheva, 1973). Our results also demonstrated that there was no significant difference with regards to pollen production between different reproductive branches (Table 2). On the other hand, it was reported that environmental factors such as low temperature during reproductive phase could limit fertility of pollen grains. In this study, to evaluate the stability of male sterility of the selected plants, a more-refined screening technique, including pollen production and pollen viability were employed in two years flowering seasons. Additional tests were made by tripping male sterile flowers that had matured in the subsequent two years. If any conflict in score was found, the plant was relisted as a partial male sterile. Our results showed that 10 out of 16 plants previously rated as fully sterile had pollens in their flowers and were reclassified as partially sterile. This may be caused by the variation in sterility expression, with partial fertility occurring to various degrees. Barnes and Garboucheva (1973) proposed that continuous variation of pollen production in cytoplasmic male sterile plants could be explained by a dose effect of fertility restoration genes. Consideration of these factors can easily be influenced by environmental effects. All 10 partial sterile plants were excluded and the remaining four fully sterile plants were designed as *ms1-ms4*, and plus two male sterile plants with multi-pistil, labeled as *M-fon1* and *M-fon2*. Based on the pollen production method, those plants repeatedly evaluated

Table 1. List of nineteen alfalfa varieties in this study and their codes

Code	Variety	Code	Variety
1	Tianshui	11	Jindera
2	Gannong No.3	12	Gannong No.2
3	Graze401+z	13	Giant
4	Golden queen	14	Ganza 27
5	Gongnong No.1	15	Algonguin
6	Qingyang	16	Emperor
7	Hexi	17	Dingxi
8	Gannong No.1	18	Longdong
9	Big west	19	Zhonglan No.1
10	Longzhong		

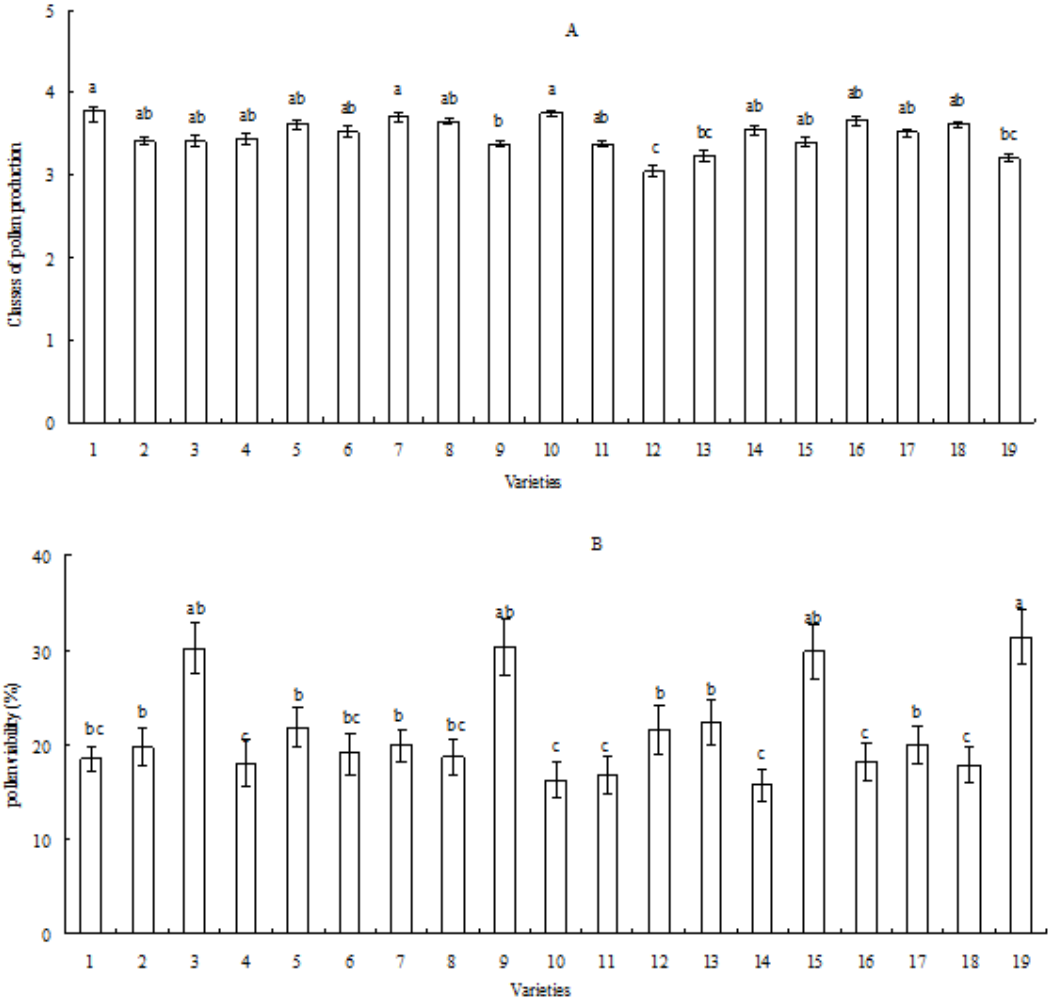

Fig 1. Comparisons of pollen production (A) and pollen viability (B) between 19 alfalfa varieties . Note: The numbers of 19 alfalfa varieties are as same as in Table 1

as fully sterile by flower tripping were also concurrently tested by staining, which indicated that this technique is useful in identifying the mutant type defective in anther dehiscence (*ms2*: Fig. 4, C).

Complete male sterile and multi-pistil male sterile mutants in alfalfa

The reproduction process to produce the microgametophytes in plant is mainly controlled by nuclear genes, so gene mutations, environmental factors and chemicals can lead to male sterility by preventing these genes from being expressed or by causing the genes to be expressed in an abnormal way (Wellmer and Riechmann, 2011). Six spontaneous male sterile mutants were identified in this study from varieties Graze401+z (*ms1, M-fon1* and *M-fon2*), Longzhong (*ms2*) and Big west (*ms3* and *ms4*), in which four mutants (*ms1-ms4*) dehiscenced no pollen grains. For *ms1-ms4*, the male gametophytic function was impaired but the female gametophyte was intact and functional during investigations from 2009-2011. These mutant types were similar to male sterile mutants found in rice (Maekawa et al., 1997) and *Arabidopsis thaliana* (Dawson et al., 1999), indicating that male sterility could occur in different plant species in natural

Table 2. Analysis of variations in pollen production and pollen variability in 19 alfalfa varieties

Variations	Source	df	F	P
Pollen production	Between groups	18	20.754	<0.001
	Within groups	931		
Pollen viability	Between groups	18	5.208	<0.001
	Within groups	931		
PP of reproductive branch	Between groups	4	0.529	0.589
	Within groups	931		

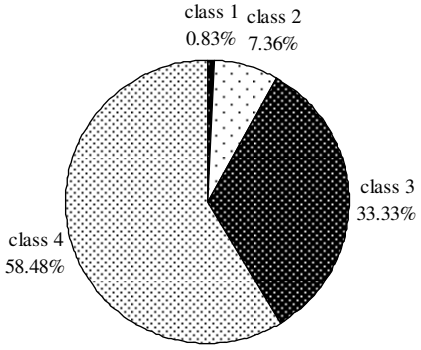

Fig 2. Percentage distributions of four classes of pollen production screened in 19 alfalfa varieties

ecosystems in the same way. Recently, environmentally sensitive male-sterile mutants such as *ms8* and *msp* in soybean (Frasch et al., 2011), thermo-sensitive genetic male-sterile mutants and cytoplasmic male sterile lines in rice (Lee et al., 2005) were reported. However, there are only two main types of male sterile mutants characterized in alfalfa. One of these types, the complete male-sterile mutant was caused by genetic factors. Childers and McLennan (1960) described a complete male sterile clone 20DRC both under greenhouse and field conditions, and the male sterility of clone 20DRC was inherited as a recessive characteristic. It was reported that cytoplasmic male sterility in alfalfa was characterized by variations in sterility expression, with partial fertility occurring to various degrees (Davis and Greenblatt, 1967; Bradner and Childers, 1968; Pedersen and Stucker, 1969). The abnormality of these new male-sterile mutants had to be considered as a new source of mutation to understand male reproduction development in *Medicago sativa* L. Further genetic analysis and gene loci for male sterility using F_1 and F_2 segregation populations are in progress. Spontaneous mutation of the multi-pistil phenomena in a seeding plant is an important genetic resource for crop breeding, and similar mutants have been reported in maize (*Zea mays* L.) (Postlethwait and Nelson, 1964), oat *(Avena sativa* L.) (Bruckner and Hanna, 1990), rye (*Secale cereale* L.) (Malyshev et al., 2001) and transgenic *Medicago truncatula* (Nair et al., 2008). In addition, Hanna and Bashaw (1987) proposed that the type of multi-stigma and ovary in one floret was possibly a sign of apomixis, and the research of apomixis would contribute to establish a one line breeding system for alfalfa. Therefore, mutants *M-fon1* and *M-fon2* may provide different types of mutant with other multi-pistil crops, such as rice and sorghum, which have the potential to increase production (Li et al., 2006). On the other hand, the female sterile character of the *M-fon1* mutant showed a higher degree of degradation in stigma receptivity which would hinder the superiority of male sterility in cross-breeding. However, it may provide another way to discover the male and female fertility strategy for sexual reproduction (Barrett, 2002). The reproductive mechanisms of the multi-pistil male sterile mutants need further studies to explore these questions. The male sterile mutants were useful to explore the genetic mechanisms and the regulation of genes related to male fertility in alfalfa (Rosellini et al., 2001). Although male sterility is important in obtaining heterosis, there is no widespread exploitation of male sterility in alfalfa breeding. Therefore, the male sterile mutants could provide the basic materials for carrying out seed production in breeding programmes. The male sterile mutants (*ms1-ms4, M-fon1 and M-fon2*) obtained here and their pollination observations could potentially provide the database for alfalfa breeding. The genetic analysis and resulting selection to obtain hereditary stable male sterile lines will be useful in alfalfa hybrid seed production in the future.

Materials and methods

Study sites and plant materials

This study was carried out in Lanzhou University Ecological Research Area (Linze Experiment Station, 39º21'N, 100º07' E, altitude 1367 m) in the middle of the Hexi Corridor, northwest China. This area has an arid climate, characterized by dry, hot summers, cold winters, and plenty of sunshine. The annual precipitation is 117 mm, annual evaporation is 2390 mm. In this site, flowering time of alfalfa is from mid-May to late July and pods become ripe in the autumn (Wang et al., 2009). Nineteen alfalfa varieties of the official alfalfa Chinese breeding programme, with wide genetic diversity were studied (Table 1). Seeds of nineteen alfalfa varieties were obtained from the Grassland Research Institute, Chinese Academy of Agricultural Science at Hohhot, Inner Mongolia. All varieties were sowed in a field plot, with a complete randomized block design in April 2008. Each plot contained three rows 5 m long and 0.42 m wide, with a space of 0.84 m

Fig 3. Morphology of male sterile mutants found in alfalfa showed no pollen grains and multi-pistil. (A) no tripping, shriveled anthers, no pollen; (B) no tripping, healthy anther, contains pollen; (C) tripping, shriveled anther without cracking, no pollen; (D) tripping, anther dehiscence, numerous pollen grains. (E) two pistils in the floret of a no-pollen plant; (F) three pistils in the floret of a no-pollen.

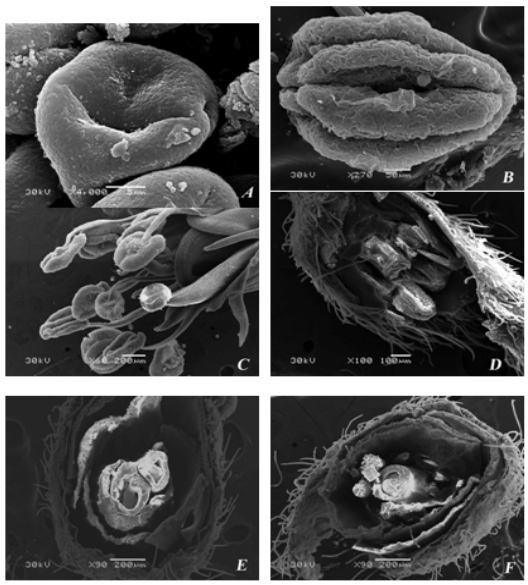

Fig 4. Scanning electron microscopy of six male sterile mutants. (A) pollen develop abortion mutant *ms1*; B) anther of the male sterile mutant *ms2*, no pollen observed; (C) anther dehiscence mutant *ms2*; (D) anther develop abortion mutant *ms3*; (E) two pistils of the male sterile mutant *M-fon1*; (F) three pistils of the male sterile mutant *M-fon2*.

between adjacent plots. There were 5 plots and 10 replicates of each variety per plot. Irrigation and pest control practices were instigated when needed.

Determination of pollen production and pollen viability

The term "natural male sterile mutant" in alfalfa plants refers to the absence of functional pollen grains, which is primarily determined by pollen production (PP) and pollen viability (PV). Male-sterility is often considered a developmental flaw. Pollen production in individual plants of the 19 alfalfa varieties was visually scored from May to July in 2009. Each variety had 50 replicates, therefore in each variety, 10 individuals were randomly tested and in each individual, 5 florets from different branches were tested. Pollen production was divided into 4 classes: class 1 = no visible pollen, plants

displayed no pollen when flower was tripped; class 2 = traces of pollen, plants dehisced only a trace of pollen when the flower was tripped; class 3 = moderate amounts of pollen, plants dehisced moderate amounts of pollen when the flower was tripped; and class 4 = much pollen dehisced when the flower was tripped (Pedersen and Barnes, 1973). Plants with pollen production class 1 were considered to express the male sterile traits, whereas plants scoring class 3-4 were considered as male fertile. Sixteen male sterile plants with pollen production class 1 were labeled in 2009. The 2, 3, 5-triphenyl tetrazolium chloride (TTC) method was used to determine pollen viability (PV) of each individual plant according to the modified method of Lansac et al. (1994). A slide containing 1-2 drops of 0.5% TTC was evenly mixed with fresh pollen and kept at 35℃ for 25 minutes, then pollen grains were examined using a light microscope. The percentage of viable pollen was estimated by counting the number of red coloured pollen grains compared to the total number of grains counted in each treatment. The total number of grains counted was more than 300 each time. The pollen production of the 16 labeled male sterile plants was tested again from May to July in 2010 and 2011. Six robust male sterile mutants were identified as having no functional pollen and designated as *ms1-ms4*. Two multi-pistil male sterile mutants were also found. These were designated as *M-fonl* and *M-fon2*.

Scanning electron microscope (SEM) observation

Floret samples of the normal plants and the male sterile mutants were primarily fixed in 3% glutaraldehyde and 0.2 M phosphate buffer (pH 7.0) for 2-24 hours. They were then washed in 0.2 M phosphate buffer three times, and then post-fixed in 1% osmium tetraoxide overnight. After fixation, all samples were washed in deionized water and dehydrated with 50%, 70%, 80%, 90%, 95% and 100% ethanol for SEM. The dehydrated materials were removed from the pure alcohol and then put into 10% tert-butyl alcohol for two hours. The 10% tert-butyl alcohol was then removed and replaced with fresh 10% tert-butyl alcohol and left for a further two hours. Then samples were freeze dried using TEOL JFD-310 freeze dried device under -5 ^{o}C overnight. Then the material was pasted to an observation board for metal spraying, and placed in a Scanning Electron Microscope (JSM-680LA) for scanning observation and photographs.

Statistical analyses

Means ± SE were calculated for pollen production, pollen viability and pollen production of different reproductive branches. One-way Analysis of Variance (ANOVA) was used to determine differences of alfalfa pollen production and pollen viability within varieties and among varieties, as well as pollen production between different reproductive branches, using SPSS (version 13.0, SPSS Inc., IL, USA) software package.

Acknowledgements

We thank Heather Rumble from Royal Holloway University of Landon for helping revised the manuscript. This work was financially supported by National Basic Research Program of China (973 Program) (No. 2007CB108904) and Fundamental Research Funds for the Central Universities of Lanzhou University (lzujbky-2010-1).

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