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Characterization of nine alfalfa varieties for differences in ovule numbers and ovule sterility

Xiaojuan Wang[1], Xiaoxia Li[1], Jingwen Zhang[1], Guanghui Feng[1], Shuzhen Zhang[1], Lichun Huang[1], Renying Zhuo[2], Liang Jin[1*]

[1]School of Pastoral Agriculture Science and Technology, Lanzhou University, P.O. Box 61, Lanzhou 730020, China
[2]Key Lab of Tree Genomics, The Research Institute of Subtropical of Forest, Chinese Academy of Forest, Fuyang 311400, China

***Corresponding author: liangjin@lzu.edu.cn**

Abstract

Alfalfa (*Medicago sativa* L.) is one of the most important forage species worldwide. In order to discover the mechanism of low seed production, two cytological experiments were carried out to investigate ovule numbers per floret and ovule fertility among nine different alfalfa varieties. Results showed that there was significant difference in ovule numbers per floret among the nine alfalfa varieties ($P<0.05$), and the number of ovules per floret ranged from 7 to 13. The highest ovule number was Huangyangzhen while the lowest one was Ladak. Results also showed that there was significant difference in ovule fertility among different alfalfa varieties ($P<0.05$), and the average percentage of ovule sterility was 34.90%. The highest percentage of ovule sterility was observed in Russia with 49.07%, while the lowest percentage sterility was 25.20% in Zhungeer. Alfalfa seed set under artificial-pollination showed a significantly linear correlation with ovules per floret ($P<0.05$) but no significant correlation under self-pollination treatments. The results showed that the fertile ovules per pistil accounted for 51-75% with total ovules per pistil, but the percentages of actual seed yields with potential seed yields of the nine varieties were only 2.49-6.06%, which suggested that ovule sterility maybe just one of the limiting factors for alfalfa seed production. Our results indicated that female fertility of alfalfa was remained in the nature reproductive ability, which was probably due to the breeding selection program of alfalfa that was mainly focused on yield or quality, but seldom on seed production.

Keywords: fertility, floret, *Medicago sativa* L., seed production, seed set.
Abbreviations: ANOVA, analysis of variance.

Introduction

Alfalfa (*Medicago sativa* L., 2n=4x=32) is one of the most important perennial legume crops and a superior source of forage due to its high nutritional quality and herbage yield (Abusuwar and Bakri, 2009; Keivani et al., 2010; Wang et al., 2011). Seed yield of alfalfa is important in determining the effective distribution of new cultivars to farmers (Bolaños-Aguilar et al., 2002; Li and Brummer, 2009). Seed per pod is an important index which relate to the number of ovules per pod and the success rates of seed set. The ovule number reflects the potential number of seeds that could be produced per pod (Krarup and David, 1970; Tischner et al., 2003). It has been demonstrated that the potential seed yield of a crop depended on the total number of ovules per unit area presented at flowering time, and this is usually high for all forage species (Daphne et al., 1999). However, the realized seed potentials of forage species are very low, especially compared to the major grain crops (Falcinelli, 1999; Lorenzetti, 1993; Thomas and Pasumarty, 1996). Lorenzetti (1993) reported that the realized seed potential of alfalfa was only 4% of the potential seed yield. According to this result, it is very interesting that why seed set is generally low while alfalfa has such high potential for phytomass production and such large numbers of flowers presented in sexual propagation (Iannucci et al., 2002)? Until now, the seed potential production ability is still poorly realized in alfalfa. Thus, a major research effort should be directed at exploiting and manipulating reproductive mechanisms in forage legumes for the purpose of increasing realized seed yield efficiency to meet the seed production potential (Falcinelli, 1999; Hossain et al., 2010). In order to breed high seed production cultivars, plants with a high number of ovules per flower maybe could result in maximum seeds per pod. It has been demonstrated that the relationship between ovule number and seed production have practical significance in alfalfa breeding program (Ilić and Đukić, 2006). The main reason for low seed productions in other genetic variations have been found to be ovule sterilities, in which there was significant negative correlation between the percentage of sterile ovules and seeds per pod (Capomaccio et al., 2009; Charlesworth, 1989; Wiens et al., 1989). In alfalfa, ovule sterility associated with deposition of callose in the nucellus has been reported (Dzyubenko and Vyshniakova, 1995) and that could contribute to a low ovule to seed ratio (Rosellini et al., 1998). Thus, checking ovule fertility in parental genotypes selected for development of varieties could help breeders obtain good seed yielding cultivars. Few studies have been focused on improving seed yield and so alfalfa could be considered similar to be wild for seed set traits. The overall goal of this research was to characterize nine alfalfa varieties by ovule number and ovule sterility to discover the mechanisms of low seed production. We conducted two cytological experiments here. The objective of the first experiment was to investigate the ovule numbers per floret among the nine alfalfa varieties. The objective of the sec-

Table 1. Code, names and origins of 9 alfalfa varieties studied

Code	Subspecies and varietas	Germplasm source	Geographical origins
1	*M. sativa* subsp. *falcata*	Liaoning	Liaoning province in northeast China
2	*M. sativa* subsp. *falcata*	Humeng	Humeng of Inner Mongolia in north China
3	*M. sativa* subsp. *sativa*	Zhungeer	Qinghai province in northwest China
4	*M. sativa* subsp. *sativa*	Huangyangzhen	Wuwei of Gansu province in northwest China
5	*M. sativa* subsp. *sativa*	Ladak	Ladakh province of Kashmir in north India
6	*M. sativa* subsp. *sativa*	Hungarian	Hungary
7	*M. sativa* var. *media*	American	America
8	*M. sativa* subsp. *caerulea*	Russian	Russia
9	*M. sativa* subsp. *falcata*	Tibetan	Tibet in southwest China

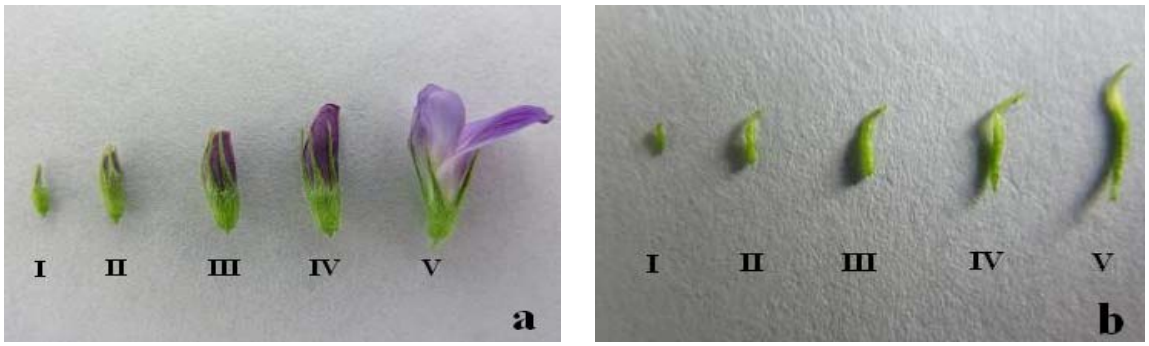

Fig 1. Flower (a) and pistil (b) developmental stages used for cytological observation. I) calyx enwraps the petals, II) petal appears between calyxs, but do not yet exceed sepal, III) the length of petals exceed calyx more than 2 mm, but the keel is still enwrapped by vexillas, IV) keel has appeared in the vexilla, but the vexilla has not yet turned up, V) flowers open, the vexilla turned up, the keel has been exposed between the wings

ond experiment was to determine the ovule fertility among different alfalfa varieties.

Methods and materials

Study site and plant materials

This study was carried out in Lanzhou University Ecological Research Area (Linze Experiment Station, 39º21'N, 100º07' E, altitude 1367m) in the middle of the Hexi Corridor, Northwest China from 2006 to 2008. Seeds of nine alfalfa varieties belonging to *M. sativa* subsp. *falcata*, *M. sativa* subsp. *sativa*, *M. sativa* subsp. *caerulea* and *M. sativa* var. *media* were obtained from Grassland Research Institute, Chinese Academy of Agricultural Science (Table 1). All varieties were sowed in the field in April 2006 with a complete randomized block design. Each plot contained three rows, each of 5 m long, 0.42 m apart, with a space of 0.84 m between adjacent plots. Each variety had five replicates. The trials were conducted across 2 years (2007 and 2008), that is, the first and second year of re-growth. Irrigation and pest control practices were in accordance with the plants recommended.

Paraffin sections of alfalfa pistil

In this study site, the flowering time of alfalfa was from mid-May to late July and pods became ripe in the autumn (Wang et al., 2009). Flower bud development period was divided into five stages according to Liu's method (1996): 1) calyx enwraps the petals, 2) petal appears between calyxs, but do not yet exceed sepal, 3) the length of petals exceed calyx more than 2 mm, but the keel is still enwrapped by vexillas, 4) keel has appeared in the vexilla, but the vexilla has not yet turned up, and 5) flowers open, the vexilla turned up and the keel has been exposed between the wings (Fig. 1a). The florets in different developmental stages of the nine alfalfa varieties were fixed in ethanol-acetic acid (3:1) for approximately 30 min and then stored in 70% ethanol until used. Pistils were dissected from the florets (Fig. 1b). After dehydration by 70%, 80%, 90%, 95% and 100% ethanol, samples were embedded in wax and then made into paraffin section. For each ovule developmental stage, several sections with a thickness of 8-10μm were stained with hematoxylin/eosin and observed under a light microscope. For each stage, 30 flowers of each variety were studied and photomicrographs were taken.

Ovule number determination

Florets of each variety were sampled randomly to count the ovule numbers under dissecting microscope. Each alfalfa variety had 30 replicates.

Ovule sterility investigation

The substance known as "callose" has been observed in plant cells by virtue of its affinity for certain dye and by its characteristic solubility behavior. Water-soluble aniline blue has been known to stain callose a clear blue. According to the method of Rosellini et al. (1998), florets were sampled at the fifth stage of flowering and fixed in 70% ethanol: acetic acid (3:1) for at least 24 hours. The pistils were then dissected, cleared in 8N NaOH for 5 hours, stained overnight in 0.1% aniline blue in 0.1M K_3PO_4, mounted in the staining solution and gently squashed under a coverslip. Fluorescence microscopy was performed and the percentage of callosized

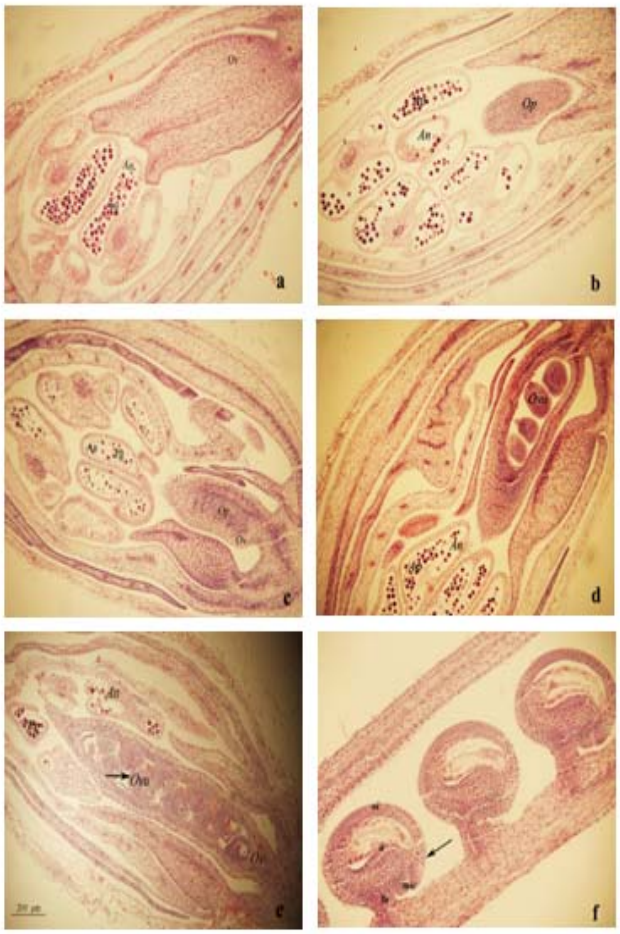

Fig 2. Longitudinal section of alfalfa young carpel showing the ovule development. a) pollen grains (Pg) in anthers (An) and ovary (Ov); b) ovule initiation with the first ovule primordium (Op) with mitotic activity in meristematic regions; c) the ovule originates as a small protuberance; d) the formation of ovules (Ovu), e) the maturity of the ovules, f) the outer integument (oi), inner integument (ii), funiculus (fu) and micropyle (mic) of ovule.

ovules within the ovary was recorded in nine alfalfa varieties according to the method of Barcaccia et al. (1996).

Seed set and actual seed production

The florets per variety were selected randomly and masked for away bees' pollination until pods appeared. Each variety had 300 replicates. At the same time, the florets per variety were selected to cross by artificial emasculation in this case. Each variety had 100 replicates. All the pods under bees' pollination and artificial pollination were then collected to evaluate the seed number per pod using the following equation: Seed set = pod % × seed numbers per pod. Plants of each variety were then sampled to evaluate seed production. Each alfalfa variety had 10 replicates. The number of reproductive branches per plant, number of inflorescence per reproductive branch, number of pods per inflorescence and the number of seeds per inflorescence were calculated. The potential seed yield and realized seed yield were calculated according to the method of Lorenzetti (1993).

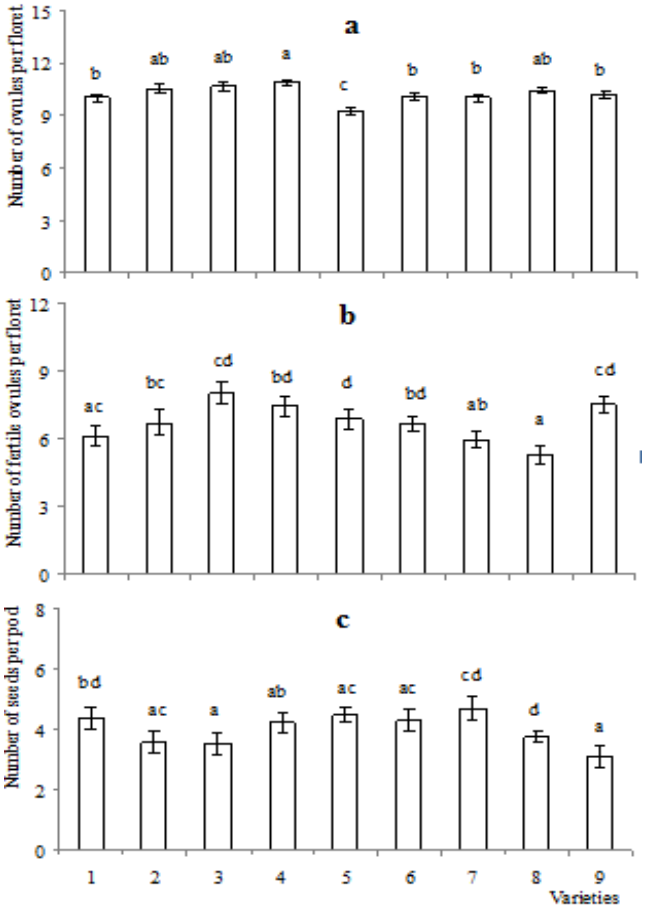

Fig 3. Results of numbers of ovules per floret (a), fertile ovules per floret (b) and number of seeds per pod (c) among the nine alfalfa varieties (±SE). The name of the numbers for varieties was showed in Table 1.

Data analysis

All the datasets were checked for normality prior to further analysis, and none of them were significantly different from a normal distribution, so no data transformation was required. Means and variance of the ovule number per floret, fertile ovules and seeds per pod were calculated using SPSS (V13.0). One-way ANOVAs were used to test differences among alfalfa varieties for the ovule numbers, fertile ovules and seeds per pod. Correlation analyses were performed to determine the potential relationship between ovule numbers, fertility of ovules and seed set.

Results

Ovule development

According to the cytological observation, the ovule first appears as a mound of tissue, and one or more archesporial cells beneath the epidermis could be distinguished by their large nuclei and dense cytoplasm (Fig. 2 a, b). At first the ovules of alfalfa arise in two rows, one on each side of the ventral suture. With the rapid lengthening of the carpals, the ovules soon become widely separated in the rows (Fig. 2 c, d). In the fifth developmental stage, micropyle and integument (inner and outer) of ovules were visible, and the

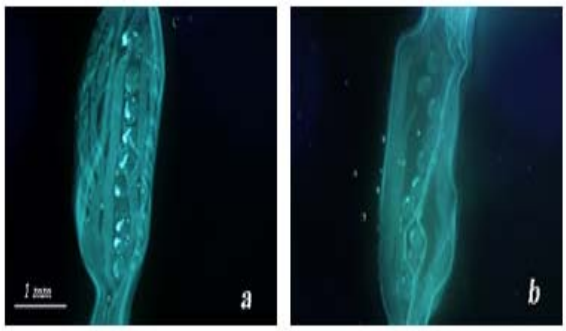

Fig 4. Photo micrographs of cleared pistils showing callose deposition in the ovules of alfalfa plants. a) callose deposition observed on all ovules; b) callose deposition observed on some ovules

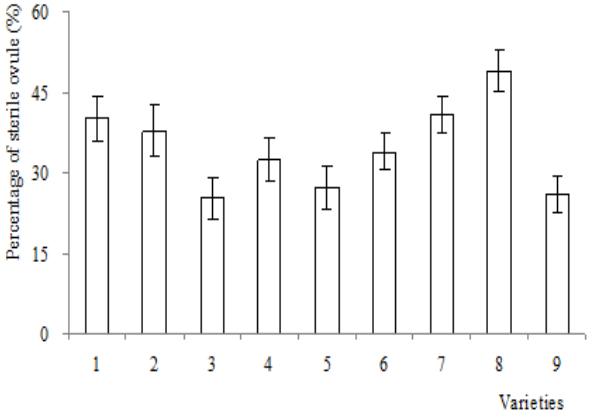

Fig 5. Comparisons of the percentage of sterile ovule per floret among the nine alfalfa varieties (±SE). The name of the numbers for varieties was showed in Table 1.

callus deposition in ovule could be observed in the mature pistil (Fig. 2 e). Similar to the results of Reeves (1930), we also observed that the young ovule was orthotropous until it came into contact with the dorsal wall of the carpel and then it began to curve. This curvature was usually towards the base of the ovary (Fig. 2 f). The comparisons among these nine varieties found that there were no significant differences in ovule development periods among the nine alfalfa varieties.

Genetic variation of ovule numbers

The numbers of ovules per ovary, fertile ovules per floret and the numbers of seeds per pod among the nine alfalfa varieties varied between the different genetic groups (Fig. 3 a, b, c). And there was significant difference in ovule numbers per floret and seeds per pod among the nine alfalfa varieties (P<0.05). The mean value of ovule number for each variety showed that the minimum and maximum numbers of ovules per floret were 7 and 13 respectively. The highest number was in Huangyangzhen (*M. sativa* subsp. *sativa*), with 10.93±0.97 while the lowest number was in Ladak (*M. sativa* subsp. *sativa*), with 9.26±1.10 (Fig. 3 a).

Genetic variation of ovule sterility

The analysis of ovule fertility was conducted by observing callose deposition in the pistils of different varieties in the fifth period (Fig. 4 a, b). Callosized ovules were found in all populations, and the average percentage of ovule sterility per floret was 34.90%. These results showed that there was significant difference in ovule fertility among alfalfa varieties (P<0.05). The highest percentage of ovule sterility was observed in Russia (*M. sativa* subsp. *caerulea*), with 49.07% sterility, whilst the lowest ovule sterility was observed in Zhungeer (*M. sativa* subsp. *sativa*), with 25.20% (Fig. 5), which indicates that alfalfa ovule sterility varies among the nine varieties.

Correlations between ovule traits and seed set

According to the correlation analysis, the seed set under artificial-pollination showed a significantly linear correlation with ovules per floret (r=0.4631, P<0.05) and no correlation under self-pollination. The fertile ovules per pistil accounted for 51-75% of total ovules per pistil, but the percentages of actual seed yields with potential seed yields of 9 alfalfa varieties were only 2.49-6.06%. Our results indicate that the actual seed yield of alfalfa is low and the sterility of ovules is possibly just one of the limiting factors for maximum realized seed production.

Discussion

There has been remarkable interest in the molecular and genetic analysis of ovule development in recent years (Kelley and Gasser, 2009; Losa et al., 2010; Schneitz, 1999). Our investigation demonstrated that there were no consistent differences in the cytological characteristics of ovule development in the nine varieties belong to *M. sativa* subsp. *falcata*, *M. sativa* subsp. *sativa, M. sativa* subsp. *caerulea* and *M. sativa* var. *media*. In the early stages of ovule development, the archesporium in alfalfa was not sharply delimited from other tissues, and it was, therefore, often difficult to determine with certainty whether a cell was archesporial or not. Then the ovule developed rapidly and became campylotropous with the micropyle against the funiculus (Fig. 2 f), which conformed in general to the description of *M. sativa* by Reeves (1930). The reason of this anatropous curvature may be related to unequal growth at the funicular region (Chehregani and Tanaomi, 2010) and the origin and sequence of integument formation (Bouman, 1984). Much of the present challenge was the elucidation of the molecular structure of the identified sporophytic and gametophytic genes, and the analysis of their genetic interactions. However, few female gametophyte mutations have been described to date, which most likely reflects the technical complexity of identifying and characterizing the mutations, rather than the actual number of genes involved (Capomaccio et al., 2009; Pereira et al., 1997). Number of ovules per ovary represents the potential ability of a flower to produce seeds. Thus, it appears that selection of plants with a high number of ovules per flower should result in maximum seeds per pod (Barnes and Cleveland, 1963). Several studies on the genetic control of ovule number have been initiated in *M. sativa* L. (Barnes and Cleveland, 1963; Lorenzetti, 1993), *Pisum sativum* L. (Krarup and Davis, 1970) and *Theobroma cacao* L. (Clement et al., 2003). Barnes and Cleveland (1963) found that high ovule number was controlled by four genes, three of which showed complete or nearly complete dominance, while the fourth expressed a degree of incomplete

dominance, and genetic effects of all four loci were additive. In general, the number of ovules per ovary of alfalfa ranged from 8 to 14, with a dominant number of 10 to 12. Our results also indicated that the numbers of ovules per ovary in these nine alfalfa varieties ranged from 7 to 13, and that the genetic variations of ovule numbers existing in nine different alfalfa varieties should provide basic data for selection and improvement program of seed yield in the future. Ovule sterility is an important factor in determining low seed set which has been indicated in several species (Pasumarty et al., 1993; Pereira et al., 1997; Rim et al., 1990; Seavey and Carter, 1996; Thomas and Pasumarty, 1996). However, unless an easily scorable marker is available, collecting data on ovule sterility is time consuming and is impractical to examine many plants. By associating ovule sterility with callose deposition and using a quick stain clearing technique based on callose fluorescence, the results on ovule sterility of hundreds of plants were determined in this study. Rosellini *et al*. (1998) reported that variation of percent ovule sterility among florets of a plant was relatively high but repeated sampling of the same plants over time showed that the expression of the trait was comparatively stable. Thus, different sterility genes and mechanisms may result in callose deposition within the ovule. On the basis of this, the callose could perhaps be used as a marker of sterility and a selection tool, irrespective of the genes that were involved in the different populations. Compared to the percentage of sterile ovules in *Epilobium obcordatum* at 39% (Seavey and Carter, 1996), in *Lotus corniculatus* at 10.5% (Rim et al., 1990), in *Trifolium repens* at 28-33% (Pasumarty et al., 1993), and in white clover at 37% (Thomas and Pasumarty, 1996), the ovule sterility of the nine alfalfa varieties ranged from 25.20% to 49.07%. Alfalfa is generally recognized as a very variable plant, and the variability is probably due to the fact that the plant has been artificially bred and cultivated for a long time, and thus many different varieties and cultivars have been produced (Julier et al., 2000; Reeves, 1930). Thomas and Pasumarty (1996) have proposed ovule sterility as an important component of low seed set in *Trifolium repens*. In fact, callose was normally deposited around the megaspore mother cell and degraded after non-fuctional megaspore degeneration (Barcaccia et al., 1996). It is possible that meiosis is blocked and callose is not degraded but deposited further, perhaps to act as a molecular filter preventing pollen tube-attracting substances from guiding pollen tubes to sterile ovules. The realized seed potential is very low in alfalfa. The ratio of seed per ovule in the field was estimated about 0.08, of which the major cause of this deficit was the low number of seeds produced per pod in the floret. In general, no more than 5 ovules per pod could become seeds (Lorenzetti, 1993). In our results, the correlation between the seed set under artificial-pollination showed a significantly linear correlation with ovules per floret ($P<0.05$) but not under self-pollination. The fertile ovules per pistil accounted for 51-75% of total ovules per pistil, but the percentages of actual seed yield with potential seed yield of the 9 alfalfa varieties were only 2.49-6.06%. Significant negative correlation was found between the percentage of sterile ovules and seeds per pod in most alfalfa populations. Checking ovule fertility in parental genotypes selected for variety development could help breeders obtain good seed yielding cultivars. A new genetic ovule sterility trait has been demonstrated in a plant from the alfalfa cultivar Blazer XL, named B17, which has 81% ovules displaying heavy callose deposition at the time of anthesis and low female fertility (Rosellini et al., 1998). The number of seeds per pod was negatively correlated with the percentage of sterile ovules. Also the pod-floret ratio was significantly negatively correlated with sterility.

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