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Quantitative ovule sterility in *Medicago sativa*

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Abstract Ovule sterility was found to be associated with callose deposition in B17, a plant with low fertility from the alfalfa cv Blazer XL. The site of callose deposition, which began during embryo-sac development and affected 81% of the ovules in mature florets, at random positions in the ovary, appeared to be the embryo-sac wall or the integumentary tapetum. The fertile ovules of B17 transmitted the ovule-sterility trait to the progenies, thereby demonstrating a sporophytic genetic control. B17 was crossed with P13, a Peruvian plant with 5% callosized ovules, to generate reciprocal F_1 populations, and an F_1 plant (91% callosized ovules) was used to obtain the backcross populations. B17 was also crossed to unrelated, highly fertile, plants. S_1 progenies from B17 and P13 were also studied. All the progeny populations displayed continuous variation for the percentage of sterile ovules, supporting a polygenic control. Narrow-sense heritability estimated by offspring-midparent regression was 0.85. Reduced transmission of the sterility trait through the pollen is hypothesized to explain the difference between reciprocal crosses. Six progeny plants showing 100% callosized ovules proved to be female-sterile. Ovule sterility could be an important component of the generally observed low realized seed potential in alfalfa.

Key words Alfalfa · Embryo sac · Female sterility · Callose · Heritability

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Introduction

A low seed/ovule ratio is a general characteristic of outcrossing species (Wiens 1984; Guth and Weller 1986; Wiens et al. 1987; Bawa and Buckley 1989; Seavey and Carter 1996). Forage legumes are no exception (Dobrowsky and Grant 1980; Stephenson 1984; Lorenzetti 1993; Thomas 1996).

The genetic load of recessive alleles has been implicated in the determination of embryo abortion (Meinke and Sussex 1979; Wiens 1984; Marshall and Ludlam 1989; Wiens et al. 1989; Burbidge and James 1991; Pasumarty et al. 1993; Johnson et al. 1994; Helenurm and Schaal 1996), but the role of prefertilization ovule sterility has rarely been taken into account.

Ovule sterility has been reported in several species and sterility genes can act gametophytically (Pereira et al. 1997, and references therein) or sporophytically (Klucher et al. 1996, and references therein).

Sterility of ovules with normal morphology has ben documented in several species. In *Epilobium obcordatum* (Seavey and Carter 1996) an average of 39% of the ovules were sterile. In a *Lotus corniculatus* plant 10.5% sterile ovules were counted (Rim et al. 1990). Pasumarty et al. (1993) examined the ovules of four *Trifolium repens* cultivars and reported that 28–33% of the ovules per carpel were sterile. In a study conducted on a single white clover plant, a minimum of 37% of the ovules were found to be sterile (Thomas and Pasumarty 1996).

Kolyasnikova (1985) reported ovule sterility associated with deposition of callose in the nucellus of *Medicago sativa* plants. It was highly variable in the cv Vela: one plant was completely fertile and no plant completely sterile. Oryol et al. (1986) also found ovule sterility associated with the presence of callose in the chalazal portion of the embryo sac in *M. sativa*, *M. varia* and ten other *Medicago* species. More recently,

Dzyubenko and Vyshniakova (1995) reported that callose deposition occurred in the endothelium cell walls in sterile ovules of the alfalfa cv Tibetskaya.

Callose (β1,3 and β1,4 glucan) has been associated with the loss of viability of ageing, unfertilized, ovules (reviewed in Dumas and Knox 1983). Callose fluorescence has been observed, beginning 4 days before and ending 3 days after anthesis, in and around mature embryo sacs of *Torenia fournieri* (Tiwari 1982), in the endothelium walls in *Arabidopsis thaliana* during embryogenesis (Webb and Gunning 1994), and in the endothelium *of Petunia hybrida* beginning at pollination and ending after fertilization (Esser 1963). Callose has also recently been observed in a nucellus-like region of sterile ant-9 mutant ovules of *A. thaliana* (Elliot et al. 1996).

Barcaccia et al. (1996) described the callose deposition pattern during megasporogenesis in diploid *Medicago falcata* and *Medicago coerulea*. The callose is deposited as a micropylar cap in the megaspore mother cell and as cell plates between dyad, triad and tetrad cells. The degradation of callose starts after meiosis and has never been detected from the binucleate embryo-sac stage onwards.

It has been proposed that the role of callose in angiosperm sporogenesis is that of temporarily isolating the meiocyte and spores from the surrounding tissue by acting as a molecular filter (reviewed in Kapil and Tiwary 1978).

We have investigated the inheritance of an ovule sterility trait by determining callose deposition in mature ovules of alfalfa.

Materials and methods

The following tetraploid alfalfa plants and progeny populations were used in this study.

Single plants

B17: plant from the cultivar Blazer XL with low cross-fertility (1.10 seeds floret^{-1}, Bingham, unpublished).
P13: plant from a Peruvian accession (PI536535) with a cross-fertility of 4.7 seeds floret^{-1} in un-emasculated crosses with the plant BP described below.
F_1-s: plant randomly selected from the B17 × P13 F_1 population.
BCP-195: plant from the F_1-s × P13 backcross population.
BCP-257: plant from the F_1-s × P13 backcross population.
BP: plant from the cv Blazer XL selected for high cross-fertility (eight seeds floret^{-1}, Bingham, unpublished).
6-4ms: cytoplasmic male-sterile plant selected by E. T. Bingham from the cv Saranac, characterized by good cross-fertility (5.31 seeds floret^{-1} in crosses with BP).

Progeny populations

S_1 B17: 15 plants.
S_1 P13: 18 plants.
F_1 B17 × P13: 40 plants.
F_1 P13 × B17: 34 plants.
Backcross B17 × F_1-s: 25 plants.
Backcross F_1-s × P13: 25 plants.
F_1 6-4ms × B17: 22 plants.
F_1 6-4ms × BP: 23 plants.

All crosses except B17 × BP were made following suction emasculation. All the plants were grown in pots in a greenhouse at Madison, Wisconsin, USA, from November 1996 to July 1997 under continuous light.

Florets were sampled at flowering. For the plants listed above, the number of florets, from at least three random racemes per plant, ranged from 19 to 40, and the corresponding number of ovules from 183 to 413. For plants sampled more than once (see Table 1), the samples were taken on successive re-growths about 40 days apart. For progeny populations, the numbers of plants and ovules examined per plant are reported in Table 3.

Flowers were fixed in 70% ethanol : acetic acid 3 : 1 for at least 24 h. Then the pistils were dissected, cleared in 8 N NaOH for 5 h, stained overnight in 0.1% aniline blue in 0.1 M K_3PO_4 (Martin 1959), mounted in the staining solution and gently squashed under a coverslip. Fluorescence microscopy was performed according to Barcaccia et al. (1996).

In an attempt to obtain data on the timing of callose deposition, 20 floral buds (about 6-mm long, tips of the petals just emerging from the calix), 20 mature and 20 senescing un-fertilized florets of P13 and B17 were sampled and the percentage of callosized ovules per pistil determined.

The percentage of callosized ovules in relation to the position of the ovules within the ovary was recorded for plants B17, F1-s, BP and 6-4ms; ovule positions were numbered starting from the distal (stigmatic) end of the ovary.

The percentage of ovules with callose deposition was determined per floret, per plant, and per progeny population. For each progeny population, the frequency distribution of the percentage of callosized ovules per plant was studied. The significance of the differences between the means of the reciprocal F_1 populations and between the means of the two backcross populations was determined by the Wilcoxon test.

The mean of the progenies was regressed on the mean of their parents to obtain an estimate of narrow-sense heritability (Levings and Dudley 1963).

In vitro pollen germination of B17, P13 and BP was determined on a 200-grain basis according to Rosellini et al. (1994).

Results

Callose deposition and ovule sterility

In the highly female-sterile plant B17, callose appeared to surround the entire embryo sac of a variable number of ovules per floret at the time of anthesis. Florets with 100% callosized ovules were frequent (Fig. 1 A), while florets containing no callosized ovules were not found. Based on the shape of the fluorescent area, the embryo-sac wall or the integumentary tapetum, or both, are the sites of callose deposition. Embryo-sac shape and size appear normal (Fig. 1).

Despite a degree of variation in the amount and pattern of callose deposition (Fig. 1 D–F), ovules were separable into two distinct classes: callosized and non-callosized.

The pattern of callose deposition found in ovules of the other six plants examined (Table 1) was the same as

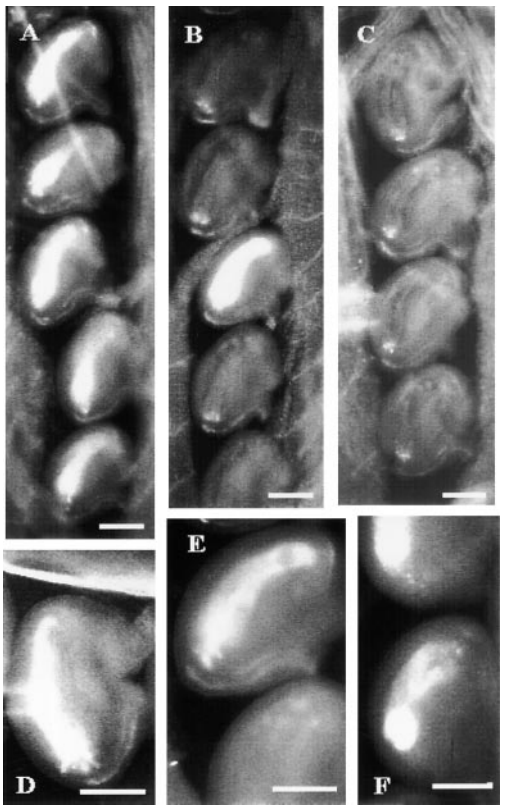

Fig. 1A–F Photo-micrographs of cleared pistils showing callose deposition in the ovules of the plant B17. **A–C**, details of ovaries were 100% (**A**), one (**B**) and no (**C**) callosized ovules are seen; **D–E**, different degrees and patterns of callose deposition. Bars = 100 μ

Table 1 Percentage of callosized ovules in seven alfalfa plants

Plants	1st sample	2nd sample	3rd sample	Mean	Range per floret
B17	85	80	79	81	40–100
P13	0	11	–	5	0–90
F_1-s	94	89	–	91	60–100
BCP195	99	98	–	98	90–100
BCP257	6	5	–	6	0–22
BP	5	–	–	–	0–18
6-4ms	9	–	–	–	0–30

observed in B17. All plants had at least some callosized ovules, and F_1-s and BCP-195 showed a higher callosized ovules percentage than their parent B17.

Variation of the percent ovule sterility among florets of an alfalfa plant was relatively high (Table 1), but repeated sampling of the same plants over time showed that the expression of the trait was comparatively stable.

On the basis of ovule positions 1 to 10 (Fig. 2) no evidence of a relationship between the position of the ovule in the ovary and the percentage sterility was found in B17, F_1-s, BP and 6-4ms.

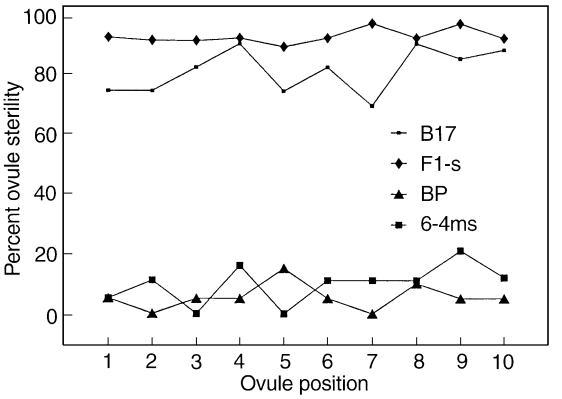

Fig. 2 Percentage of callosized ovules in relation to ovule position within the ovary. Ovules are numbered starting at the stigmatic end of the ovary. Positions 11 to 13 were not included due to a low number of observations. Forty florets were used for F1-s, 20 for the other plants

The association of ovule sterility with callose deposition was demonstrated using six plants from the backcross population B17 × F1-s. These plants proved to be completely female-sterile when 19–84 florets per plant were cross-pollinated by hand using BP as the pollen source. They showed 100% callosized ovules. Therefore callosized ovules were assumed to be sterile and non-callosized ovules fertile, though sterility unrelated to callose cannot be ruled out. In fact, B17 produces 1.10 seeds floret^{-1} when hand crossed (Bingham, unpublished), and the seed set expected if all non-callosized ovules are fertile is 1.90, based on 10.0 average ovules floret^{-1} and 81% callosized ovules. Ovule sterility, lack of fertilization, and abortion all contribute to lower the seed set. In any case, callosized ovules will be referred to as sterile.

An ovule sterility trait associated with retarded integument development has been described in alfalfa by Bingham and Hawkins-Pfeiffer (1984). This trait affects ovule development and morphology, whereas the one described above seems to affect embryo-sac viability.

The finding that immature ovules from floral buds of B17 and P13 at the approximate stage of a 4–8-nucleated embryo sac showed percentages of callosized ovules similar to those of mature ovules (Table 2) is evidence that deposition begins during embryo-sac development. There was no marked difference in the percentage of callosized ovules between immature, mature and senescing florets of B17 or P13 (Table 2). Weaker callose fluorescence, which spread to the xylem and the chalazal end of the ovule (data not shown), rendered calculating the percentage of sterile ovules uncertain in senescing un-pollinated pistils of B17. The appearance of callose and its progressive spread in un-pollinated ageing ovules was related to the loss of viability for pollen tubes and ovule senescence (reviewed in Dumas and Knox 1983).

Table 2 Percentage of callosized ovules in floral buds, and mature and senescing flowers of B17 and P13

Plant	Flower age (from 20 florets)	Ovules examined	Callosized ovules %
B17	Flower buds	191	91
	Mature flowers	208	81
P13	Flower buds	196	6
	Mature flowers	195	4
	Senescing flowers	187	5

Table 3 Percentage of callosized ovules observed in nine alfalfa progeny populations

Progeny population	Number of plants	Ovules examined per plant	Callosized ovules %	
			Progeny mean	Midparent
S_1 B17	15	10–129[b]	75	81
S_1 P13	18	3–100[b]	11	5
F_1 B17 × P13	40	121–215[a]	46	43
F_1 P13 × B17	34	40–218[a]	31	43
BC B17 × F_1-s	25	153–249[a]	77	86
BC F_1-s × P13	25	126–235[a]	61	48
F_1 B17 × BP	19	17–199[b]	35	43
F_1 6-4ms × B17	22	38–197[b]	35	45
F_1 6-4ms × BP	23	37–106[b]	11	7

[a] From at least three racemes per plant
[b] From one raceme per plant

Genetic behaviour of ovule sterility

Table 3 reports mean callosized ovule percentages of the progenies examined, midparent values, and sample sizes. The distribution frequency of the progenies are presented in Figs. 3 and 4. Plants B17 and P13, contrasting with respect to callosized ovule percentages, produced clearly distinct S_1 progenies (Fig. 3 A). The fact that the fertile ovules of B17 transmitted ovule sterility (callosized ovules) to the progenies is proof of sporophytic genetic control. There was a degree of variation in both S_1 populations, indicating that segregation most likely occurred in both parents. In particular, P13 (5% sterility) produced two S_1 plants with 25% sterile ovules, and B17 (81% sterility) one S_1 plant with only 53% sterile ovules.

Both reciprocal F_1 populations displayed continuous variation (Fig. 3 B–C), thereby indicating polygenic control of the callosized-ovules trait.

Sterility was significantly higher in the B17 × P13 F_1 population than in the reciprocal P13 × B17 population (Table 3) ($P = 0.008$), and their distribution was also different (Fig. 3 B–C). Careful suction emasculation usually results in very low or no selfing, and the low self-fertility of P13 makes it very unlikely that the 0–10% class in the P13 × B17 F_1 population comprises

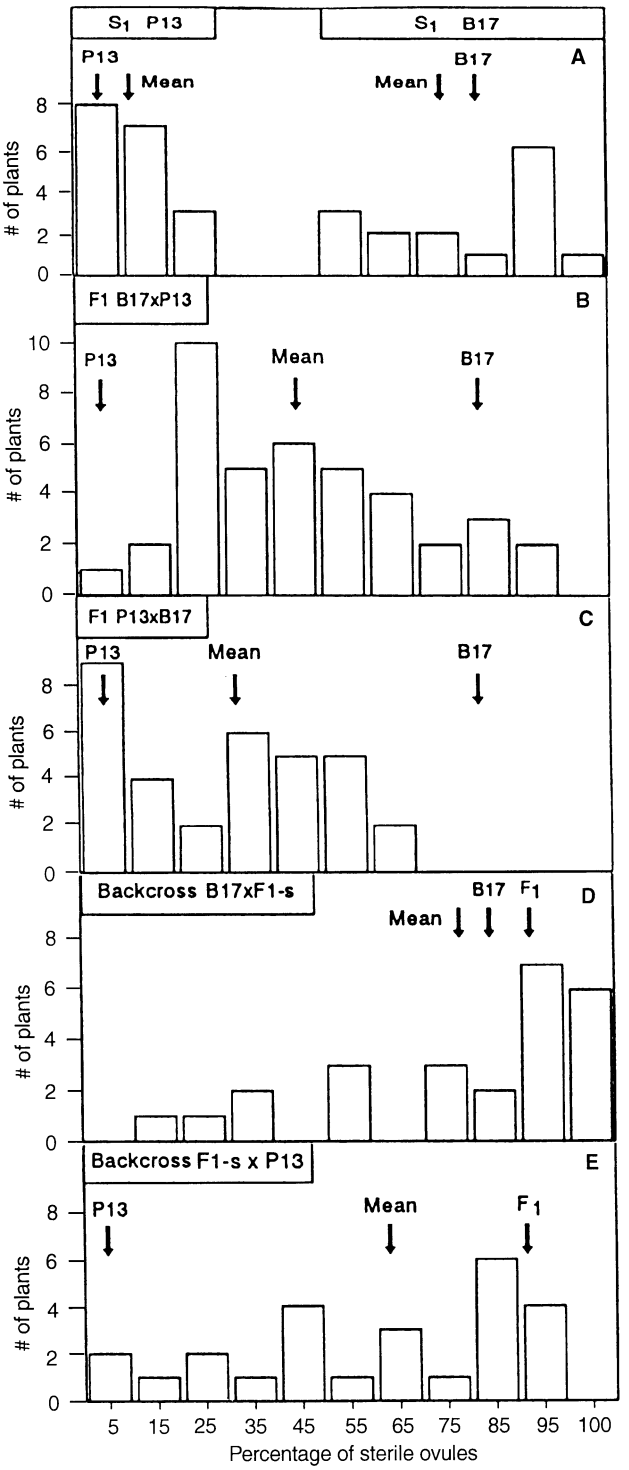

Fig. 3A–E Frequency distributions of the percentages of callosized ovules per plant in the progenies of B17 and P13

selfed plants. In any case, no plants with sterility higher than 70% were observed in the P13 × B17 F_1 population, while 17.5% of the plants were more than 70% sterile in the B17 × P13 F_1 population. An interaction between nuclear and cytoplasmic factors could account

for the difference between reciprocal crosses, but reduced transmission of the sterility trait through the pollen of B17 is more likely, considering that B17 pollen production was low. Microgametogenesis was not examined. There was no difference in the in vitro pollen germination of B17 (81.5%) and P13 (81.5%). If microgametophytic selection was a factor, it must have occurred before pollen-grain maturation.

The comparison between the two backcross populations, both in B17 cytoplasm, gives an indication of the effect of nuclear genes *per se* (Fig. 3 D–E): 75% and 25% B17 genes determined a mean sterility of 77% and 61%, respectively (which differ at $P = 0.022$). The mean of the B17 × F1-s population was slightly lower than the parental values, while the mean of the F1-s × P13 backcross population was intermediate between the parents, again an indication of reduced transmission of sterility through F1-s pollen. Transgressive segregation was marked in B17 × F_1-s; in particular, 24% of the plants were 100% sterile, and 32% were clearly less sterile than either parent. The parents may have been heterozygous for different sterility loci. No plants were 100% sterile in F_1-s x P13, and there was no transgressive segregation.

The distribution frequency of the B17 × BP progeny is presented in Fig. 4 A. The single 100% sterile plant of this progeny was considered to be from selfing, which implies 5% selfing in the un-emasculated hand-cross, a reasonable expectation based on our previous experience. One plant out of 15 was 100% sterile in B17 S_1 progeny (Fig. 3A). The progeny mean, calculated excluding the putative selfed plant, was lower than that of the B17 × P13 population (Fig. 3 B), which suggests that BP complemented the ovule sterility of B17 more than P13. Both female fertility and in vitro pollen germinability were higher in BP than in P13 (8.0 vs 4.7 seeds floret^{-1} and 94.5 vs 85.5%).

The cross 6-4ms × B17 (Fig. 4 B) produced a progeny with an average sterility similar to that of the cross P13 × B17. When 6–4ms was crossed with BP a low-sterility progeny was obtained and there were only a few plants with relatively high sterility (Fig. 4 C).

The regression of the mean ovule sterility of offspring on midparents (based on data reported in Table 3) provided a narrow-sense heritability estimate of 0.85, with a standard error of 0.11. Despite the fact that some dominance effects contribute to this estimate in autotetraploids (Levings and Dudley 1963) additive gene action is prevalent.

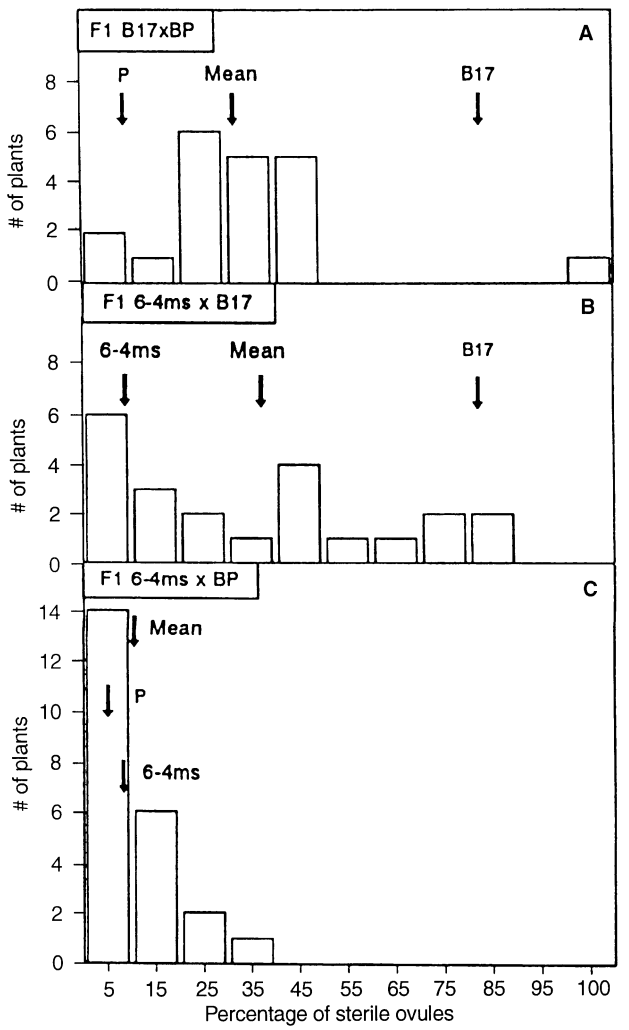

Fig. 4A–C Frequency distributions of the percentage of callosized ovules per plant in the progenies of B17 × BP, 6-4ms × B17 and 6-4ms × BP

Discussion and conclusions

Unless an easily scorable marker is available, collecting data on ovule sterility is time consuming and it is impractical to examine many plants. By associating ovule sterility with callose deposition we were able to examine more than 200 plants and 25 000 ovules in the present study.

The examination of whole mount preparations of cleared pistils did not allow a fine determination of the site and pattern of callose deposition, which will require the study of sectioned material.

We showed that, due to genetic factors in alfalfa, few-to-many ovules can be sterile and unavailable for fertilization. Lack of fertilization of apparently normal ovules has been documented in both alfalfa (Cooper et al. 1937; Cooper and Brink 1940; Sayers and Murphy 1966; Sangduen et al. 1983; Rosellini et al. 1994) and other species (Guth and Weller 1986; Krebs and Hancock 1991; Montalvo 1992). In alfalfa, Sayers and Murphy (1966) reported that some ovules were by-passed by pollen tubes, and Cooper et al. (1937) noted that unfertilized ovules contained apparently normally formed embryo sacs. More recently, Oryol and Kazachkovskaya (1991) have proposed embryo-sac

heterogeneity in un-pollinated ovaries as the cause of low seed set. We suggest that failure of ovules to attract a pollen tube and to become seeds is mainly due to genetic sterility.

Continuous variation in the progeny populations studied, transmission through the fertile ovules, and high heritability of ovule sterility all demonstrate a quantitative sporophytic additive inheritance. Control by more than one gene is likely but, in autotetraploid alfalfa, five genotypic classes are possible for a single additive locus ($A_1A_1A_1A_1$, $A_1A_1A_1A_2$, $A_1A_1A_2A_2$, $A_1A_2A_2A_2$, $A_2A_2A_2A_2$), and environmental effects, incomplete penetrance and variable expressivity can determine continuous variation even with a single gene. Environmental effects should not be important as shown by the stability of a plant over time.

The position of callosized ovules within the ovary was random in our study, demostrating that sterility does not depend on a different physiological condition of proximal vs distal ovules that could be caused by inefficient metabolite transport.

One of the mechanisms underlying genetic ovule sterility in alfalfa could be the partitioning of assimilates between vegetative organs and developing pistils. The gene(s) involved might affect the allocation of assimilates to the reproductive organs and so cause starvation of the developing ovules. Early deposition of callose could be a way of excluding starving ovules from the competition for pollen tubes.

Because of the reduced pollen production of B17, a comparative study of pollen and ovule formation will be conducted aimed at ascertaining whether there is any correlation between ovule fertility and pollen production, since the same mechanism could cause both pollen and ovule sterility.

Other ovule-sterility mechanisms not associated with callose may be present in alfalfa, where seed-ovule ratios are about 0.08 in the field (Lorenzetti 1993). As even high-seed-set plants (BP and 6-4ms) presented some sterile ovules it would seem that ovule sterility, irrespective of whether it is associated with callose, can be an important component of the low realization of seed potential in alfalfa. Thomas (1996) has proposed ovule sterility as an important component of low seed set in *Trifolium repens*. Alfalfa is a polysomic polyploid that can carry a high genetic load of deleterious alleles (Demarly 1979; Jones and Bingham 1995), and its perenniality should permit the accumulation of a genetic load for fertility (Klekowski 1988; Kimbeng 1996). Moreover, since very little effort has been devoted to seed trait improvement, the occurrence of high ovule sterility in cultivated alfalfa is not surprising.

High narrow-sense heritability suggests that selection for ovule fertility should be easy, but that this translates to an improvement of seed production in the field has yet to be demonstrated. Cytological investigations and the study of the trait in *Medicago* populations are necessary to elucidate the mechanisms of ovule sterility and its relevance for breeding.

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References

Barcaccia G, Mazzucato A, Falcinelli M, Veronesi F (1996) Callose localization in cell walls during meiotic and apomeiotic megasporogenesis in diploid alfalfa. Caryologia 49:45–56

Bawa KS, Buckley DP (1989) Seed:ovule ratios, selective seed abortion, and mating system in Leguminosae. In: Stirton CH, Zarucchi JL (eds) Advances in legume biology. Monogr Syst Bot Missouri Bot Gard 29:243–262

Bingham ET, Hawkins-Pfeiffer J (1984) Female sterility in alfalfa due to a recessive trait retarding integment development. J Hered 75:231–233

Burbidge AH, James SH (1991) Postzygotic seed abortion in the genetic system of Stylidium (Angiospermae: Stylidiaceae). J Hered 82:319–328

Cooper DC, Brink RA (1940) Partial self-incompatibility and the collapse of fertile ovules as factors affecting seed formation in alfalfa. J Agric Res 60:453–472

Cooper DC, Brink RA, Albrecht HR (1937) Embryo mortality in relation to seed formation in alfalfa (Medicago sativa). Am J Bot 24:203–213

Demarly Y (1979) The concept of linkat. In: Zeven AC, Van Harten AM (eds) Proc Conf Broadening Genetic Base of Crops. Centre for Agriculture Publishing and Documentation, Wageningen, The Netherlands, pp 257–265

Dobrowsky S, Grant WF (1980) An investigation into the mechanism for reduced seed yield in *Lotus corniculatus*. Theor Appl Genet 57:157–160

Dumas C, Knox RB (1983) Callose and determination of pistil viability and incompatibility. Theor Appl Genet 67:1–10

Dzyubenko NI, Vyshniakova MA (1995) Intraspecific variability of seed productivity character in alfalfa. Proc 3rd Int Herbage Seed Conf, Halle, Germany, June 23–28, pp 162–166

Elliot RC, Betzner AS, Huttner E, Oakes MP, Tucker WQJ, Gerentes D, Perez P, Smyth D (1996) Aintegumenta, an Apetala2-like gene of *Arabidopsis* with pleiotropic roles in ovule development and floral organ growth. Plant Cell 8:155–168

Esser K (1963) Bildung und Abbau von Callose in den Samenanlagen der *Petunia hybrida*. Z Bot 51:32–51

Guth CJ, Weller SG (1986) Pollination, fertilization and ovule abortion in *Oxalis magnifica*. Am J Bot 73:246–253

Helenrum K, Schaal BA (1996) Genetic load, nutrient limitation, and seed production in *Lupinus texensis* (Fabaceae). Am J Bot 83:1585–1595

Jones JS, Bingham ET (1995) Inbreeding depression in alfalfa and cross-pollinated crops. Plant Breed Rev 13:209–233

Johnson S, Liu CM, Hedley CL, Wang TL (1994) An analysis of seed development in *Pisum sativum*. XVII. The isolation of mutants defective in embryo development. J Exp Bot 45:1503–1511

Kapil RN, Tiwari SC (1978) Plant embryological investigations and fluorescence microscopy: an assessment of integration. Int Rev Cytol 53:291–331

Kimbeng CA (1996) Genetic experiments in alfalfa (*Medicago sativa* L.): fertility, genetic load and S1 forage yield performance in original and improved two-allele autotetraploid populations

of alfalfa. PhD thesis, University of Wisonsin-Madison DA # 96 13353

Klekowski EJ Jr (1988) Mutation, developmental selection, and plant evolution. Columbia University Press, New York

Klucher KM, Chow E, Reiser L, Fischer RL (1996) The *AINTEGUMENTA gene of Arabidopsis* required for ovule and female gametophyte development is reated to the floral homeotic gene *APETALA2*. Plant Cell 8 : 137–153

Kolyasnikova NL (1985) Determining the fertility of alfalfa by means of fluorescence microscopy. Bot Issledovaniya na Urale 25

Krebs SL, Hancock JF (1991) Embryonic genetic load in the highbush blueberry, *Vaccinium corimbosum* (Ericaceae). Am J Bot 78 : 1427–1437

Levings CS, Dudley JW (1963) Evaluation of certain mating designs for estimation of genetic variance in autotetraploid alfalfa. Crop Sci 3 : 532–535

Lorenzetti F (1993) Achieving potential herbage seed yield in species of temperate regions. Proc XVII Int Grassland Cong 1621–1628

Marshall C, Ludlam D (1989) The pattern of abortion of developing seeds in *Lolium perenne* L. Ann Bot 63 : 19–27

Martin FW (1959) Staining and observing pollen tubes in the style by means of fluorescence. Stain Technol 34 : 125–128

Meinke DW, Sussex IM (1979) Isolation and characterization of six embryo-lethal mutants in *Arabidopsis thaliana*. Dev Biol 72 : 62–72

Montalvo AM (1992) Relative success of self- and out-cross pollen comparing mixed- and single-donor pollinations in *Aquilegia coerulea*. Evolution 46 : 1181–1198

Oryol LI, Kazachkovskaya EB (1991) Embryological heterogeneity as the cause of reduction in seed productivity in *Medicago sativa* (Fabaceae). Bot Zhurl 76 : 161–172

Oryol LI, Kostantinova LN, Ogorodnikova VF, Dzyubenko NI (1986) The fertility of lucerne ovules and methods for its evaluation. Sbornik Nauchnykh Trudov po Prikladnoi Botanike, Genetike i Selektsii 99 : 10–17

Pereira TNS, Ilarslan H, Palmer RG (1997) Genetic and cytological analyses of three lethal ovule mutants in soybean (*Glycine max*; *Leguminosae*). Genome 40 : 273–285

Pasumarty SV, Matsumura T Higuchi S, Yamada T (1993) Causes of low seed set in white clover (*Trifolium repens* L). Grass Forage Sci 48 : 79–83

Rim YW, Beuselinck PR, McGraw RL, Somers DA (1990) Megagametophyte development in *Lotus corniculatus*, *L. conimbricensis*, and their protoplast fusion hybrid. Am J Bot 77 : 1084–1094

Rosellini D, Veronesi F, Falcinelli M (1994) Recurrent selection for microgametophytic vigor in alfalfa and correlated responses at the sporophytic level. Crop Sci 34 : 933–936

Sangduen N, Sorensen EL, Liang GH (1983) Pollen germination and pollen tube growth following self-pollination and intra- and inter-specific pollination of *Medicago* species. Euphytica 32 : 527–534

Sayers ER, Murphy RP (1966) Seed set in alfalfa as related to pollen tube growth, fertilization frequency and post-fertilization ovule abortion. Crop Sci 6 : 365–368

Seavey SR, Carter SK (1996) Ovule fates in *Epilobium obcordatum* (*Onagraceae*). Amer J Bot 83 : 316–325

Stephenson AG (1984) The regulation of maternal investment in an indeterminate flowering plant (*Lotus corniculatus* L). Ecology 65 : 113–121

Thomas RG (1996) Limitations to seed set in white clover (*Trifolium repens* L.). I. Preliminary observations. J Appl Seed Prod 14 : 59–66

Thomas RG, Pasumarty SV (1996) Limitations to seed set in white clover (*Trifolium repens* L.). II. The influence of light intensity on floral development. J Appl Seed Prod 14 : 67–72

Tiwari SC (1982) Callose in the walls of mature embryo sac of *Torenia fournieri*. Protoplasma 110 : 1–4

Webb MC, Gunning BES (1994) Cell biology of embryo sac development in *Arabidopsis*. In: Williams EG, Clarke AE, Knox RB (eds) Genetic control of self-incompatibility and reproductive development in flowering plants. Mol Biol Plants 2 : 461–485

Wiens D (1984) Ovule survivorship, brood size, life history, breeding systems and reproductive success in plants. Oecologia 64 : 47–53

Wiens D, Calvin CL, Wilson CA, Davern CI, Frank D, Seavey SR (1987) Reproductive success, spontaneous embryo abortion and genetic load in flowering plants. Oecologia 71 : 501–509

Wiens D, Nickrent DL, Davern CI, Calvin CL, Vivrette NJ (1989) Developmental failure and loss of reproductive capacity in the rare palaeoendemic shrub *Dedeckera eurekensis*. Nature 338 : 65–67