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POLSKA AKADEMIA NAUK

ACTA BIOLOGICA CRACOVIENSIA Series Botanica 53/1: 96–101, 2011
DOI: 10.2478/v10182-011-0013-4

CYTOEMBRYOLOGICAL ANALYSIS OF CAUSES FOR POOR SEED SET IN ALFALFA (*MEDICAGO SATIVA* L.)

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Received February 3, 2011; revision accepted April 21, 2011

Poor seed set is a limiting factor in alfalfa *br*eeding, as it slows the selection response. One strategy used to overcome this problem is to search for mutations of inflorescence morphology. Long-peduncle (*lp*), branched-raceme (*br*) and top-flowering (*tf*) inflorescence mutations increase the number of flowers per inflorescence, but they do not improve seed set per flower. Here we assessed pollen tube growth in styles of those inflorescence mutants and we observed embryo and endosperm development in seeds 1 to 16 days after pollination (DAP). The number of pollen tubes penetrating the style and the ovary was similar in all tested mutants and in the reference cultivar Radius. At 2 DAP, fertilized ovules were 2.7–3.9 times less numerous in certain inflorescence mutants than in the short-raceme cv. Radius. Ovule degeneration progressed at 2–4 DAP in all analyzed plants. Most ovules were not properly developed in the control cultivar (62%), nor in the forms with mutated inflorescence morphology (69–86%). The number of seeds per pod was lowest in the *tf* form despite its having the highest number of ovules per ovary. It appears that the number of ovules per pistil is not a crucial factor in seed set in alfalfa when fertilization efficiency is very low. Both poor fertilization and gradual ovule degeneration were factors causing poor seed set in the investigated alfalfa genotypes.

Key words: *Medicago sativa*, seed set, pollen tubes, embryo development, inflorescence mutants.

INTRODUCTION

Alfalfa (*Medicago sativa* L.) is a major fodder plant in Poland and worldwide, but the selection response of this species is slow (Doliński and Hefny, 2006). This is due primarily to poor seed set, which hinders breeding and limits the extent of its cultivation (Bolanos-Aguilar et al., 2000). The search for genes determining traits affecting seed formation is of great importance for the production of new forms of alfalfa with higher productivity. One approach is genetic analysis of *M. truncatula*, a species closely related to alfalfa, which is autogamous and shows good seed set (Cook, 1999). Another approach is to expand the range of variation by the use of mutations, especially inflorescence morphology mutations (Staszewski et al., 1990, 1992). Long-peduncle, branched-raceme and top-flowering inflorescence mutants produced more flowers per inflorescence and altered plant habits, both important for higher yields, but those mutations did not improve seed set per flower.

The aim of this study was to assess pollen germination and pollen tube growth, and to analyze embryo and endosperm development in seeds produced in alfalfa plants of three inflorescence mutations: long peduncle (*lp*), branched raceme (*br*) and top flowering (*tf*). We compared the results for these mutations to those of a short-raceme cultivar, Radius.

MATERIAL AND METHODS

This study used three spontaneous inflorescence mutants of alfalfa (*Medicago sativa* L.): *lp*, a mutant with long-peduncle inflorescences derived from cv. Vernal (characterized by a longer torus and a higher number of flowers in the raceme than in short-peduncle plants); *br*, a mutant with branched-raceme inflorescences obtained by crossing cv. Radius with French genotypes (inflorescences with morphology unusual for alfalfa, as they not only form secondary and further branches but also pro-

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PL ISSN 0001-5296
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duce more flowers than the racemes of short-peduncle plants do); and *tf*, a mutant with top-flowering inflorescences derived by crossing cv. Radius with French genotypes (inflorescences are formed at the top of the main shoot, which results in termination of growth of the main stem after completion of flowering). The control material consisted of plants of a short-raceme cultivar, Radius (a medium-early, well-wintering cultivar, with good regrowth potential and relatively resistant to lodging).

The experiment was done in a greenhouse of the Department of Genetics and Plant Breeding, Poznań University of Life Sciences. Plant material originated from the Institute of Acclimatization and Plant Breeding, Radzików, Poland. In March, seeds were sown in boxes with horticultural peat mixed with sand (3:1). Then seedlings 10 cm long were transplanted individually to pots (Ø18 cm) with substrate containing *Rhizobium* bacteria (horticultural peat mixed with soil from an experimental plot). After 6 weeks the plants were transferred to pots (28 cm) permitting unimpeded development of the root system. Plants were watered with tap water and fed with liquid fertilizer (Florovit) once a week. After ground temperatures no longer dropped below 0°C, the plants were kept outdoors through the end of September.

Flowers were pollinated with pollen of plants belonging to the same mutation line or cultivar. Material for analysis of pollen tube growth in pistil tissues was fixed 12, 24 and 48 h after tripping (i.e., squeezing the flowers gently to release the column in order to allow pollination). Material for embryogenesis observations was fixed 1, 2, 4, 8 and 16 days after tripping. Collected material was fixed for 24 h in Carnoy fixative (95% ethanol/chloroform/ice-cold acetic acid, 6:3:1) and then refrigerated in 70% ethanol. Pollen tubes were observed using fluorescence after aniline blue staining according to Martin (1959) as modified by Wojciechowski and Dyba (1979). Pistils were macerated in 1N NaOH (60°C, 2 h), rinsed three times with distilled water and stained for 24 h in aniline blue with K_3PO_4. Preparations were sealed in glycerol and observed by fluorescence microscopy. Pollen tubes were analyzed in 15 pistils of each inflorescence form. To determine ovule or seed development and embryogenesis stage at different times after pollination, ovules in pistils 1–4 days after pollination (DAP) and ovules isolated from pods at 8 DAP were observed after a modified tissue clearing procedure in methyl salicylate (Young et al., 1979). Pistils or immature seeds were dehydrated in an ethanol series (85% 1 h, 95% 1 h, 100% 2 × 30 min + 1 h) and cleared in mixtures of 100% ethanol and methyl salicylate (3:1 and 1:1) and then twice (1 h each time) in methyl salicylate. Preparations were sealed in methyl salicylate and observed under a microscope with Nomarski interference contrast. Stages of embryo development at 16 DAP were analyzed in immature seeds and embryos from fixed pods under a dissecting microscope. The analyzed material consisted of 103 pistils of the *lp* mutant, 108 pistils of the *br* mutant, 69 pistils of the *tf* mutant and 85 pistils of cv. Radius.

RESULTS

POLLEN GERMINATION AND POLLEN TUBE GROWTH

Pollen tubes were observed in pistils of all three inflorescence mutants and cv. Radius as early as 12 h after pollination. The number of pollen grains germinating on the stigma and the number of pollen tubes observed in the styles or ovaries varied between individual flowers (Tab. 1). On average there were 7.4–13.4 germinating pollen grains on the stigma (Fig. 1a) and 6.8–9.0 pollen tubes in styles. In flowers of long-peduncle (*lp*) plants we found a particularly high number of germinating pollen grains (mean 20.6) and pollen tubes growing in styles (mean 15.4) 24 h after pollination. On average there were 4.2–11.9 pollen tubes penetrating the ovary (Fig. 1b); the number of pollen tubes was higher than that only in some ovaries from *lp* and branched-raceme (*br*) inflorescences 24 h after pollination. Only a few ovules penetrated by pollen tubes appeared in top-flowering (*tf*) and *lp* inflorescences 12 h after pollination, short-raceme cv. Radius 24 h after pollination, and *br* mutants 48 h after pollination.

EMBRYOLOGICAL ANALYSIS OF OVULES IN POLLINATED FLOWERS

Pistils of the *lp* form had 10.4 ovules on average (n=46, R: 8–15), and their pods contained 3.5 seeds on average (n=57, R: 1–8). The embryos were at globular stage (8 days after pollination, DAP) or with well-developed, bent cotyledons (16 DAP, horseshoe stage). Seeds formed in 33.6% of the ovules in flowers of those plants.

Pistils from *br* inflorescences contained 9.3 ovules on average (n=48, R: 7–12) but formed pods containing 2.8 seeds (n=60, R: 0–6) with globular embryos (8 DAP) or embryos at the horseshoe, stick or torpedo stages (16 DAP). The seed set percentage for flowers of that mutant was 30.1%.

In flowers of *tf* inflorescences there were 11.0 ovules per pistil (n=45, R: 9–15) but only 2.0 seeds were formed per pod (n=24, R: 1–5). This gave the lowest percentage (18.2%) of seed set per flower among the plant forms studied here. The seeds contained globular embryos (8 DAP) or embryos at the horseshoe stage (16 DAP). The short-raceme con-

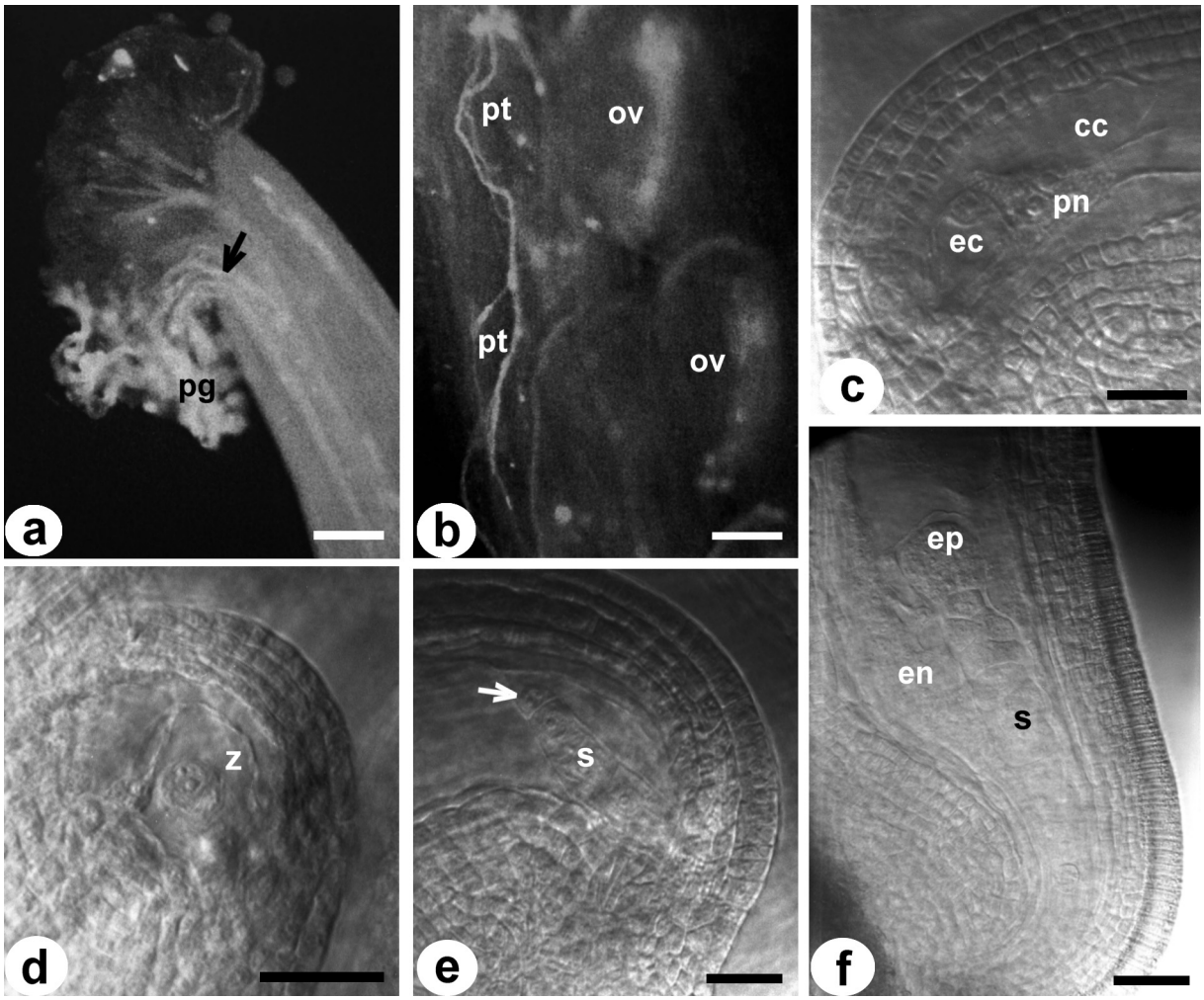

Fig. 1. Pollen tube growth (**a, b**) and early embryogenesis (**c, f**) in *Medicago sativa* pistils 24 h (**a–d**), 48 h (**e**) or 8 days (**f**) after self-pollination. (**a**) Pollen grains (pg) germinating in base of stigma. Some pollen tubes pass into the style (arrow); cv. Radius, (**b**) Pollen tubes (pt) of long-peduncle mutant growing in ovary next to ovules (ov), (**c**) Micropylar region of female gametophyte containing egg cell (ec) and polar nuclei (pn) in central cell (cc); long-peduncle mutant, (**d**) Zygote (z) at micropylar end of female gametophyte; cv. Radius; two nucleoli appear in zygote nucleus, (**e**) Several-celled proembryo and (**f**) globular embryo in immature seeds; cv. Radius; arrow in (e) and ep in (f) – embryo proper, s – suspensor, en – endosperm. Bars = 200 μm (a, b), 50 μm (c–f).

trol, cv. Radius, had 10.1 ovules per pistil on average (n=45, R: 8–12), and 2.8 seeds per pod (n=40, R: 0–9). Those seeds contained embryos at the same stages of embryogenesis as those given above, and 27.7% of the ovules in cv. Radius flowers formed seeds.

To determine the reasons for the poor seed set in all studied alfalfa forms we analyzed embryo structure and embryogenesis from 1 to 4 DAP. We found mature female gametophytes 24 h after pollination in all inflorescence mutants and in cv. Radius (Fig. 1c). One percent of the female gametophytes in *br* and *tf* forms, 9% in the *lp* form, and 18% in cv. Radius had already been fertilized and contained a zygote and 4 endosperm nuclei (Fig. 1d). A much higher number of ovules did not develop female gametophytes and showed symptoms of degeneration (flattened shape, with hollow integuments and nucellus). At 24 h after pollination, 33–51% of the ovules of the mutants had degenerated; in cv. Radius only 15% of the ovules had degenerated. At 2 DAP the pistils of plants with modified inflorescence morphology had 7–14% immature seeds and 46–57% degenerated ovules; the corresponding values for cv. Radius are 34% and 13%. At 4 DAP the inflorescence mutants showed 18–24% immature seeds and 69–86% degenerated ovules; cv. Radius had the highest num-

TABLE 1. Pollen germination and pollen tube growth in alfalfa (*Medicago sativa* L.) after self-pollination in mutants with long-peduncle inflorescences (*lp*), branched-raceme inflorescences (*br*) or top-flowering inflorescences (*tf*) and in the short-raceme cv. Radius (control). Mean values (X_m) and the range of variation (R) are given for 15 flowers per sample

Form of *M. sativa* and time after pollination	Number of germinating pollen grains on stigma		Number of pollen tubes in style		Number of pollen tubes in ovary	
	X_m	R	X_m	R	X_m	R
Short-peduncle (cv. Radius)						
12 h	13.4	6–17	8.8	0–12	6.0	0– 8
24 h	14.2	8–19	13.2	7–17	11.6	6–16
48 h	10.8	10–12	8.2	5–10	5.6	1– 9
Long-peduncle (*lp*)						
12 h	9.0	5–12	9.0	6–12	7.0	4–11
24 h	20.6	18–26	15.4	11–19	9.8	7–14
48 h	10.2	3–19	9.4	3–15	4.8	1– 8
Branched-raceme (*br*)						
12 h	7.8	3–16	6.8	2–16	6.2	1–15
24 h	14.5	6–18	13.8	2– 7	11.9	2– 5
48 h	11.2	3–25	8.2	3–14	4.8	0– 8
Top-flowering (*tf*)						
12 h	7.4	5– 9	7.0	4– 9	6.0	4– 8
24 h	6.8	4– 8	6.0	3– 8	4.2	2– 6
48 h	10.0	6–15	8.8	6–14	5.8	4–10

TABLE 2. Occurrence of immature seeds and degenerated ovules in pistils of alfalfa (*Medicago sativa* L.) 1–4 days after self-pollination in mutants with long-peduncle inflorescences (*lp*), branched-raceme inflorescences (*br*) or top-flowering inflorescences (*tf*) and in the short-raceme cv. Radius (control). Mean values (X_m), range of variation (R) and sample size (n) are given

Form of *M. sativa* and time after pollination	Number of ovules			Frequency of immature seeds		Frequency of degenerated ovules	
	X_m	R	n	X_m (%)	R	X_m (%)	R
Short-peduncle cv. Radius							
1 day	10.6	10–12	16	1.9 (18)	0–6	1.6 (15)	0–4
2 days	10.2	9–12	13	3.5 (34)	0–6	1.3 (13)	0–3
4 days	9.5	8–11	16	3.2 (34)	0–11	5.9 (62)	0–10
Long-peduncle (*lp*)							
1 day	12.6	11–15	17	1.1 (9)	0–3	6.4 (51)	2–9
2 days	9.0	8–11	11	1.3 (14)	0–4	4.9 (54)	2–8
4 days	9.2	9–13	18	1.7 (18)	0–4	7.9 (86)	2–12
Branched-raceme (*br*)							
1 day	9.9	8– 2	15	0.1 (1)	0–1	3.9 (39)	2–6
2 days	9.9	9–12	16	1.1 (11)	0–3	4.6 (46)	1–11
4 days	8.3	7–11	17	—*	–*	6.1 (73)	3–10
Top-flowering (*tf*)							
1 day	10.1	9–12	16	0.1 (1)	0–1	3.3 (33)	1–6
2 days	12.8	10–15	14	0.9 (7)	0–3	7.3 (57)	4–10
4 days	10.3	10–11	15	2.5 (24)	0–6	7.1 (69)	2–11

* no data available; abundant hairs on the ovary wall prevented observations

ber of immature seeds (34%) but the percentage of degenerated ovules increased (62%). The respective data are given in Table 2.

Early embryo development progressed at similar rates in branched-raceme (*br*), top-flowering (*tf*) and short-raceme plants (cv. Radius), although fertilization occurred slightly later in inflorescence mutants than in the control cultivar. In cv. Radius at 2 DAP we observed 2- or 4-celled proembryos and free-nuclear endosperm, or zygotes and endosperm nuclei; four nuclei were more frequent than two. In *br* and *tf* mutants at 2 DAP there were mature female gametophytes or, sporadically, female gametophytes with zygotes. At 4 DAP, several-celled embryos and free-nuclear endosperm were found in immature seeds of those two mutants (Fig. 1e). In long- peduncle (*lp*) plants and in cv. Radius, 2- or 4-celled proembryos or, less frequently, zygotes, appeared at 2 DAP. In the *lp* mutant, however, well-developed globular embryos with a protoderm also were observed at 4 DAP (Fig. 1f). At 8 DAP only slightly larger globular embryos were found in the flowers of all tested alfalfa forms.

DISCUSSION

A significant problem in alfalfa breeding is low percentage of seeds and low number of seeds per pod. Although alfalfa flowers are receptive for about 6 days and although the ovary contains 5–13 ovules, the number of set seeds is usually 2–5 per pod, (Cebrat, 1973; Jabłoński, 1973). The same inflorescence form, the top-flowering mutant, had the highest number of ovules per ovary (average 11.0) and at the same time the fewest seeds per pod (average 2.0). This means that the number of ovules per pistil is not critical to seed set in alfalfa. A genome sequencing project for *M. truncatula* (http://www.medicago.org) may open a new perspective on the genetic factors affecting seed set in alfalfa.

Our analyses of pollen germination and pollen tube growth showed that the number of pollen tubes penetrating the style and the ovary was similar in all tested mutants and cv. Radius. Despite this similarity, fertilization efficiency was lower in the top-flowering mutant, as confirmed by observations of seeds with formed embryos and endosperm.

Two days after pollination, in plants with long-peduncle inflorescences (*lp*), branched-raceme inflorescences (*br*) or top-flowering inflorescences (*tf*), immature seeds were 2.7–3.9 times less numerous than in the short-raceme cv. Radius. Also at 2 DAP there were 3.5–5.6 times more degenerated ovules in forms *lp*, *br* and *tf* than in cv. Radius. However, ovule degeneration progressed in all analyzed plants at 2–4 DAP and most ovules were not properly developed in the control cultivar (62%) or in the forms with mutated inflorescence morphology (69–86%). The end result of poor fertilization and gradual ovule degeneration was low seed set at 16 DAP. Approximately 30% of the ovules formed seeds in the short-raceme form (cv. Radius, the control), the long-peduncle form and the branched-raceme form. Seed set was much lower (18%) in the top-flowering form. Ovule sterility has been described in some species of angiosperms (Klucher et al., 1996; Pereira et al., 1997), and it is known in the Fabaceae (Johri et al., 1992). In *Medicago sativa*, ovule sterility is connected with deposition of callose in the nucellar tissue (Kolyasnikova, 1985; Rosellini et al., 1998), and this process may have been a factor in ovule degeneration in our study. Our results suggest an additional cause for poor seed set in alfalfa: low fertilization efficiency. In flowers from long-peduncle plants, for example, roughly every second ovule was degenerated and only 9–14% of the ovules contained an embryo and endosperm. It is known that receptive ovules play a significant role in the chemotropic mechanism of directing pollen tube growth in the ovary (Śnieżko and Chudzik, 2003; Chudzik et al., 2005). The large percentage of degenerated (i.e., probably physiologically inactive) ovules obviously resulted in less effective attraction of growing pollen tubes towards ovaries and ovules. We frequently observed looping and other changes in the direction of pollen tube growth in pistil tissues.

In the alfalfa inflorescence mutations we analyzed, an increased number of flowers did not result in a substantial increase of seed yield. The main reasons for this were (1) a very high rate of ovule degeneration, initiated immediately after pollination, and (2) slightly reduced fertilization efficiency. Further studies should focus on determining ovule degeneration before pollination. Finding forms with long peduncles or branched racemes that also exhibit low ovule degeneration would benefit alfalfa breeding.

ACKNOWLEDGEMENTS

We thank Prof. dr hab. Zygmunt Staszewski (Plant Breeding and Acclimatization Institute) for kindly providing alfalfa inflorescence mutants for cytoembryological analysis. The study was co-financed within the framework of the 5th edition of interdisciplinary research projects of the Poznań University of Life Sciences (formerly August Cieszkowski Agricultural University) and Adam Mickiewicz University in Poznań (project no. 2/02/WI/06).

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