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Microgametophytic selection in two alfalfa (*Medicago sativa* L.) clones

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Summary. Microgametophytic selection was investigated using two ecologically diverse autotetraploid clones of alfalfa. Several selection pressures (drying, aging, freezing, and high and low temperatures) were applied to microgametophytes at three stages of the life cycle, 1) during microsporogenesis, 2) post-anthesis, and 3) pollen tube growth. Pollen aging produced a progeny population with a greater mean plant size and a lower coefficient of variation than the control progeny. High temperature (29.5 °C) applied both during microsporogenesis and pollen tube growth resulted in progeny populations which were significantly taller and, in one case, had a larger leaf number than the control populations. In contrast, air dried pollen resulted in a progeny population which had significantly smaller character means and larger coefficients of variation than the control population. Also, low temperature (15 °C) during pollen tube growth yielded progeny with reduced branch number and a larger coefficient of variation than the control progeny. In cases where progeny derived from selected microgametophytes were found to differ from the control offspring, corresponding shifts in the reciprocal cross were not observed. For the temperature stress treatments, the lack of reciprocal differences may be related to the different temperature adaptations of the two ecotypes. These results suggest that microgametophytic selection can be effective in shifting the mean of the progeny generation; however, the results obtained will vary depending upon the selection pressure, stage of selection, and the parents used.

Key words: Alfalfa – *Medicago sativa* L. – Pollen selection

Introduction

Pollen selection and its influence on the resulting sporophytic generation has been suggested as an important evolutionary process in the angiosperms (Mulcahy 1979). The ability to apply selection pressure on pollen grains would be a useful breeding tool if 1) pollen and sporophytic responses to specific stresses were correlated, or 2) pollen competitive ability was correlated with sporophytic vigor. Specific stresses which have been correlated between the pollen and sporophyte include: temperature (Zamir et al. 1982; Herrero and Johnson 1980; Weaver et al. 1985), pathotoxin (Laughnan and Gabay 1973), salt (Sacher et al. 1983), copper and zinc (Searcy and Mulcahy 1985 a, b), ozone (Feder 1968), and herbicide (Smith and Moser 1985). Pollen vigor has also been correlated with sporophytic vigor in corn (Mulcahy 1971, 1974; Ottaviano et al. 1980). However, almost all of these studies were done with diploid plant species.

In autotetraploid species, sporophytic vigor is related to the number of inter- and intra-allelic interactions (Busbice and Wilsie 1966). Since the pollen from these species is 2x, the gametophyte retains an intra-locus interaction component of heterosis. In potato, the 2N gametes formed by first division restitution (FDR) had a higher germination percent after storage than pollen formed by second division restitution (SDR) (Simon and Peloquin 1976). The higher percent germination of the FDR pollen grains was attributed to the increased heterozygosity and epistasis maintained in the FDR microgametophytes.

For this study, two ecologically diverse autoteraploid clones of alfalfa (*Medicago sativa* L.) were used. Several selection pressures (drying, aging, freezing, and high and low temperatures) were applied to

the microgametophytes at different stages of their life cycle (during microsporogenesis, post-anthesis, and during pollen tube growth). If pollen selection proved to be effective in changing the sporophytic generation, it could theoretically have resulted from either an effective stress selection and/or selection for heterotic gametes.

The three major objectives were to 1) determine if selection at the gametophytic level could shift the average character value of the resulting progeny generation, 2) compare selection pressures applied at different stages of the microgametophyte's life cycle, and 3) distinguish which selection pressure(s) produced desired beneficial shift(s).

Materials and methods

Plant material

Seed of two alfalfa accessions, P.I. 299049 and GP W71-42 was acquired from the North Central Regional Plant Introduction Station at Ames, Iowa 50010 and from E. T. Bingham, Agronomy Dept. University of Wisconsin, Madison, WI 53706, respectively. One plant per initial population was selected and vegetatively propagated. The P.I. 299049 and GP W71-42 plants that were chosen had purple (P) and yellow (Y) flower colors, respectively, which were used as hybridization markers (see Table 1 for a comparison of the two clones). Hybrid progeny between the P and Y clones had a yellow/purple variegated flower color.

Pollen treatments and pollination procedure

Selection during microsporogenesis. One clone of both the Y and P alfalfa were placed in either a 29.5° or a 15 °C growth chamber with 16-hour days (light intensity≈325 $\mu mols^{-1}m^{-2}$). Three weeks later, the plants were moved to an approximately 22 °C greenhouse (16-hour day). Pollen was collected from 125 flowers from the Y and P clones which had undergone microsporogenesis in the low and high temperature growth chambers. Pollen was also collected from greenhouse grown Y and P clones. Flowers of an additional greenhouse grown Y and P clone were emasculated and used for hybridizations with pollen from the two temperature environments (29.5° and 15 °C) and the control greenhouse environment (22 °C). After pollination, the plants were grown in a 22 °C greenhouse for one month then an 18 °C greenhouse both with 16-hour days until seed collection.

Selection following anthesis. Freezing: On February 11, pollen was collected from 100 flowers each of the greenhouse grown P and Y clones and cross pollinations were immediately made. Vials containing the remainder of the pollen were placed in a −16 °C freezer for 42 days. The pollen was thawed and cross pollinations were repeated using the same set of plants. Seed was collected on March 28 and May 3. Aging: On June 20, pollen was collected from 200 flowers each of the greenhouse grown P and Y clones and crosses were immediately made onto greenhouse plants using a portion of this collected pollen. The remaining pollen was placed in 2 dram capped vials wrapped with Parafilm and foil, and stored at 25 °C for seven days. Crosses were repeated using the seven-day-old pollen with the same set of plants. Seed was collected on July 22 and 29. Air drying: Pollen was collected from a greenhouse grown P clone, placed in a clear glass vial, and set uncapped at 25 °C for 24 h. This air dried pollen was used in crosses with an emasculated greenhouse grown Y clone. Control Y×P crosses were made on five dates with freshly collected pollen.

Table 1. Comparison of the Purple vs. the Yellow alfalfa clones

Purple	Yellow
M. sativa subsp. *sativa*	*M. sativa* subsp. *falcata*
Origin – Mesopotamia (Iraq)	Origin – probably Russia
Purple flowers	Yellow flowers
Faster growing	Slower growing
Shorter	Taller
More branches	Fewer branches
More leaves	Fewer leaves

Selection during pollen tube growth. Pollen was collected from approximately 200 flowers of greenhouse grown Y and P clones. Reciprocal crosses were made on two emasculated greenhouse grown P and Y clones. One plant of each pair remained in the 22 °C greenhouse under 16-hour days and the others were placed into either a 29.5 °C or a 14 °C growth chamber with 16-hour days. Four days later when pod formation was evident, the growth chamber plants were put back into the 22 °C greenhouse.

Pollen germination

Pollen from the P and Y clones was germinated on Brewbaker's defined medium (Brewbaker and Kwack 1963) at three different temperatures: 15°, 22°, and 29.5 °C. Pollen germination was also tested for the pollen before and after the aging, air drying, and freezing treatments.

Progeny evaluation

Three cuttings were taken from each of the greenhouse grown seedling progeny after they reached flowering to eliminate maternal effects of seed size and germination date on plant size. The cuttings, consisting of two node stem segments lacking lateral branches, were dipped into a rooting powder (Rootone) and placed into a cell filled with 1 soil: 1 perlite. The cuttings were placed under intermittent mist for 14 days at which time the best rooted cutting from each seedling was transplanted into a soil filled Cone-Tainer (Ray Leach Cone-Tainer Nursery, Canby, OR 97013). Ninety-eight Cone-Tainers (3.81×20.32 cm) were situated in each 30.5×61.0 cm Cone-Tainer rack. For the progeny from the air drying treatment, five initial cuttings per plant were taken and four uniformly rooted plants retained. For this experiment only, the seedling genotypes were replicated.

The cuttings from the heat (29.5 °C) during microsporogenesis and heat during pollen tube growth experiments were grown in a 30 °C growth chamber for 32 and 35 days, respectively. Cuttings from the low temperature (15 °C) during microsporogenesis, freezing, aging, and low temperature (15 °C) during pollen tube growth experiments were grown in a 14 °C growth chamber for either 35 or 42 days. Cuttings from the air drying experiment were grown in a 22 °C greenhouse for 67 days. All plants were under fluorescent and incandescent lighting with a 16-hour day photoperiod and

were fertilized on a regular basis. After the plants were removed from the growth chamber or greenhouse, data was taken on the following characteristics: plant height, number of branches per plant, and total dry matter (excluding roots) per plant. For all experiments except the air drying experiment, the number of leaves per plant was also measured. Total leaf dry weight was measured for all experiments except air drying and low temperature (15 °C) during microsporogenesis. The width of the lowest branch was only measured in the air drying and low temperature (15 °C) during microsporogenesis experiments. Only those character means where the control and stress treatment means for either the P×Y or Y×P crosses were significantly different according to the *t*-test ($\alpha = 0.05$) are presented.

Results

Selection during microsporogenesis. Plant height was the only character which differed significantly between the control (22°) and high temperature (29.5 °C) selection during microsporogenesis progeny (Table 2). The mean height of the rooted cuttings of the P×Y-High temperature selection during microsporogenesis progeny was larger than that for the P×Y-Control progeny. In the reciprocal crosses, there were no significant differences between the control and stress treatment. *In vitro* percent pollen germination for P and Y pollen which had undergone microsporogenesis at 29.5 °C was not significantly different from the controls; however, the Y pollen tube growth appeared abnormal with many twisted spiraling pollen tubes.

There were no significant differences between the character means for the Y×P-Control vs the Y×P-Low temperature (15 °C) selection during microsporogenesis progeny when cuttings were grown at 14 °C for 42 days.

Selection following anthesis. Freezing: There were no significant differences between the character means for the Y×P-Fresh vs the Y×P-Frozen (−16 °C) progeny when cuttings were grown at 14 °C for 35 days. Aging: The Y×P-Aged (seven days) progeny had significantly higher branch and leaf number and total leaf and dry weight (excluding roots) than the Y×P-Control progeny when rooted cuttings were grown for five weeks at 14 °C (Table 3). In addition, the coefficients of variation for the Y×P-Aged (seven days) progeny characters were consistently lower than those for the Y×P-Control progeny. There were no significant differences between means for the reciprocal crosses. The aged (seven days) pollen had significantly reduced *in vitro* pollen germination. Mean P pollen germination decreased from 55.2% to 14.9% (a 73% reduction), and Y pollen germination decreased from 83.2% to 53.6% (a 36% reduction). Air drying: The Y×P-Control cuttings grown for 67 days in a 22 °C greenhouse had a significantly higher mean plant height, branch number, lowest branch width, and dry weight (excluding roots) than the Y×P-Dried (24 h) progeny (Table 4). The coefficients of variation were consistently larger for the Y×P-Dried progeny. Drying P pollen at 25 °C reduced the *in vitro* pollen germination from 73% to 38%. There were no P×Y offspring to test.

Selection during pollen tube growth. The P×Y-High Temperature (29.5 °C) selection during pollen tube growth progeny had a significantly greater mean num-

Table 2. Mean height and coefficient of variation ( ) of progeny resulting from treatments of 22 °C or 29.5 °C during microsporogenesis when rooted cuttings were grown for 32 days at 30 °C

Cross	Temp. during microsporogenesis (°C)	No. of progeny	Plant height (cm)
P×Y	22	77	32.8 (22) a*
P×Y	29.5	74	35.2 (20) b
Y×P	22	49	38.5 (19) a
Y×P	29.5	43	38.1 (26) a

* Means indicated by the same letter within one pair are not significantly different according to *t*-test ($\alpha = 0.05$)

Table 3. Mean number of branches, number of leaves, total leaf dry weight, total dry weight (excluding roots), and coefficients of variation ( ) of the progeny resulting from the post-anthesis pollen aging (7 days) experiment when rooted cuttings were grown for 5 weeks at 14 °C

Cross	No. of progeny	No. of branches/plant	No. of leaves/plant	Total leaf dry weight (g)/plant	Total dry weight (excluding roots) (g) plant
P×Y – fresh	30	4.8 (49) a*	25.1 (36) a	0.16 (49) a	0.33 (51) a
P×Y – aged	14	4.2 (51) a	22.0 (50) a	0.15 (55) a	0.29 (61) a
Y×P – fresh	50	3.5 (58) a	19.1 (40) a	0.15 (42) a	0.31 (45) a
Y×P – aged	70	4.5 (54) b	22.7 (37) b	0.19 (37) b	0.38 (39) b

* Means indicated by the same letter within one pair are not significantly different according to *t*-test ($\alpha = 0.05$)

Table 4. Mean plant height, number of branches, lowest branch width, total dry weight (excluding roots), and coefficients of variation ( ) of the progeny resulting from post-anthesis pollen drying (24 h) experiment when rooted cuttings were grown for 67 days in a 22 °C greenhouse

Cross	No. of progeny	Plant height (cm)	No. of branches/plant	Lowest branch width (cm)	Total dry weight (excluding roots) (g)/plant
Y×P – Control	45	37.3 (18) b*	9.6 (35) b	0.122 (8) b	0.52 (21) b
Y×P – Dried	42	32.6 (25) a	7.7 (37) a	0.111 (13) a	0.40 (37) a

* Means indicated by same letter within the column are not significantly different according to t-test ($\alpha = 0.05$)

Table 5. Mean height and number of leaves per plant and coefficients of variation ( ) of progeny resulting from temperatures of 22 °C or 29.5 °C during pollen tube growth when rooted cuttings were grown for 5 weeks at 30 °C

Cross	Temp. during pollen tube growth (C°)	No. of progeny	Plant height (cm)	No. of leaves per plant
P×Y	22	60	26.3 (23) a*	41.1 (27) a
P×Y	29.5	88	28.4 (24) b	44.7 (25) b
Y×P	22	97	24.6 (28) a	37.2 (32) a
Y×P	29.5	94	24.6 (32) a	37.0 (33) a

* Means indicated by the same letter within one pair are not significantly different according to t-test ($\alpha = 0.05$)

Table 6. Mean number of branches per plant and coefficients of variation ( ) of progeny resulting from temperatures of 15 °C or 22 °C during pollen tube growth when rooted cuttings were grown for 5 weeks at 14 °C

Cross	Temp. during pollen tube growth (C°)	No. of progeny	No. of branches per plant
P×Y	22	70	6.0 (43) a*
P×Y	15	100	5.7 (43) a
Y×P	22	112	5.0 (48) b
Y×P	15	55	4.2 (52) a

* Means indicated by the same letter within one pair are not significantly different according to t-test ($\alpha = 0.05$)

ber of leaves per plant and mean plant height than the P×Y-Control progeny when both progenies were grown for five weeks at 30 °C (Table 5). The progeny from the reciprocal cross were not significantly different for any of the characters measured. When both Y and P pollen samples were germinated *in vitro* at 29.5 °C, the P pollen tubes appeared, in general, two times longer than the Y pollen tubes.

The Y×P-Control offspring had a significantly greater branch number per plant than the Y×P-Low temperature (15 °C) selection during pollen tube growth progeny when cuttings were grown at 14 °C for five weeks (Table 6). The progeny from the reciprocal crosses were not significantly different for any of the characters measured.

Discussion

Pollen aging (seven days) was the most effective treatment producing a progeny population with an increased mean plant size and a reduced coefficient of variation than the control population. This suggests that mortality among pollen grains was not random but was at least partially dependent upon pollen genotype. In petunia, Mulcahy et al. (1982) also reported that plants derived from aged pollen were significantly heavier than plants from fresh pollen. Puri and Lehman (1965) suggest that reserve food material in the pollen, permeability of the pollen membrane, and the moisture content of the pollen may change over time, resulting in a decrease in pollen viability. Therefore, pollen grains which differ genetically for these traits would have different viability in storage. For example, in maize, *in vitro* pollen germination and tube growth after storage were differentially influenced by pollen genotype for the waxy, sugary, and shrunken loci which control carbohydrate and amino acid content and distribution in pollen grains (Pfahler and Linskens 1972).

It is also possible that the pollen aging treatment favored those alfalfa pollen grains which were more heterotic. In potato, increased pollen viability after storage at 5 °C in a closed container was attributed to increased pollen heterosis (Simon and Peloquin 1976). Busbice and Wilsie (1966) theorized that if the more heterotic gametes in alfalfa achieved fertilization, and if gametophytic and sporophytic vigor were positively correlated, that vigorous, highly heterozygous tetraploid plants would result.

High temperature (29.5 °C) applied both during microsporogenesis and pollen tube growth resulted in P×Y progeny populations which had increased height and/or leaf number than did the control population when grown at 30 °C. This increase

in size was also significant when the same progeny were grown in the greenhouse at 22 °C (data not presented) indicating that the difference in mean plant size between the two progeny populations was not dependent upon the temperature during plant growth. In these experiments, alleles conferring fitness to the Y microgametophytes subjected to heat may be associated with plant height since this characteristic increased in both heat treatments. Zamir and Vallejos (1983) suggested that sporophytic shifts resulting from a microgametophytic temperature selection scheme may be due to chromosome segments conferring different fitnesses to the haploid genomes in different environments.

In contrast to the aging (seven days) and high temperature treatments, pollen air drying (24 h) resulted in a Y×P progeny population that had significantly smaller character means and larger coefficients of variation for plant height, branch number and width, and total dry weight (excluding roots) than the control population. The reduction in pollen viability caused by the drying treatment may have been independent of pollen genotype. However, since pollen competition in the style was reduced, pollen which was less genotypically fit to compete may then have had an increased chance of achieving fertilization. The resulting Y×P-Air dried progeny population would be expected to have a greater frequency of poor plants, shifting the progeny mean downward. Thus, the increase in variability and downward shift in the progeny means of the Y×P-Air dried population may be attributed to the absence of naturally occurring stabilizing selection associated with high pollen competition. In cotton, spring wheat, and *Vigna,* when different quantities of pollen were put on the style, variation among the resulting plants was greater with limited as opposed to normal or excessive pollen application (Ter-Avanesian 1978).

Low temperature (15 °C) applied during microsporogenesis and pollen tube growth had little effect on the resulting sporophytic generation. The only mean character shift was a reduction in branch number for the Y×P-Low temperature selection during pollen tube growth population. Like in the pollen air drying experiment, this downward shift in the mean was associated with an increase in variability. These results are similar to those reported by Maisonneuve et al. (1986) in tomato. In their study, an F_2 population which had undergone low temperatures during pollen tube growth (15 °C day, 8 °C night) had a reduced average dry weight and a significantly greater variance than that of the control F_2 population (22 °C day/15 °C night). The greater variance for dry weight of the cold stressed F_2 was due to more smaller rather than bigger plants.

Microgametophytic selection at high (29.5 °C) and low (15 °C) temperatures had a greater effect when the selection pressure was applied during pollen tube growth as opposed to during microsporogenesis. This observation agrees with the results of Zamir and Vallejos (1983) in tomato. The cold temperature effect that they observed (measured as isozyme segregation) was 3.8 times higher when applied during pollen tube growth as opposed to during microsporogenesis.

Pollen freezing (−16 °C) did not result in any character shifts in the Y×P progeny. Freezer storage did not significantly reduce P pollen germination (38% to 23%). However, freezer storage did significantly reduce Y pollen germination (81% to 25%) but P×Y offspring were not available in sufficient numbers for progeny testing.

In the majority of the experiments performed, identical selection pressures were applied to the microgametophytes of the P and Y alfalfa clones and reciprocal crosses were made. In cases where the selected microgametophyte progeny were found to differ from the control offspring, there was never a corresponding shift in the reciprocal cross. Only the Y×P progeny populations resulting from aged (seven days) pollen and pollen subjected to low temperature during pollen tube growth differed significantly from the controls. High temperature applied during microsporogenesis or pollen tube growth resulted in a shift in the P×Y progeny but had no effect on the Y×P progeny.

The Y alfalfa clone of Russian origin is believed to be less adapted to high temperatures and better adapted to low temperatures than the P clone of Mesopotamian origin (Sinskaya 1950). Y pollen which had undergone microsporogenesis under high temperature had many pollen grains with abnormal germination. Additionally, Y pollen germinated *in vitro* at 29.5 °C had reduced tube length compared to the P pollen. In our experiments, high temperature appeared to be a more effective selection tool when applied to the more heat sensitive Y clone. The population from which the P clone was derived may have already undergone genetic selection for heat tolerant microgametophytes. Barnes and Cleveland (1972) suggested that alfalfa pollen tube length at high temperatures (25° and 30 °C) may be under genetic control.

The results in this paper suggest that microgametophytic selection can be effective in shifting the mean of the progeny generation; however, the selection pressure, stage of selection, and the parents used must be carefully chosen. For example, although pollen aging and air drying both resulted in a reduction of pollen viability, the effect of selection differed for the two stresses. The effect of temperature stress may be related to the characteristics of and variation within the pollen parent. Maisonneuve et al. (1986) suggested that the lack of success of pollen selection for cold tolerance in

their experiments compared to the success of the crosses reported by Zamir et al. (1982) was due to the differences between the parents used. The differences in cold tolerance between the parents Zamir et al. (1982) used were large, interspecific differences. In our work, the low and high temperatures were chosen from the work of Barnes and Cleveland (1972) and therefore, further refinement of the stress temperatures might have resulted in more effective selection pressure. Finally, since alfalfa is a highly heterozygous autotetraploid and much variation would normally be expected in the progeny, the progeny population character means had to be shifted substantially between progeny in order to be significant. In the case of the drying treatment, a significant difference was detected with only 45 nonstress and 42 stressed progeny. However, in a breeding program where larger progeny sizes would be grown, the population shifts may be significant and practically useful.

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