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MICROGAMETOPHYTIC SELECTION IN TWO ALFALFA (MEDICAGO SATIVA L.) CLONES

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MICROGAMETOPHYTIC SELECTION IN TWO ALFALFA [*MEDICAGO SATIVA* L.] CLONES

By

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ABSTRACT

MICROGAMETOPHYTIC SELECTION IN TWO ALFALFA (*MEDICAGO SATIVA* L.) CLONES

By

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Microgametophytic selection was investigated using two ecologically diverse autotetraploid clones of alfalfa. Several selection pressures (air drying, aging, freezing, and high and low temperatures) were applied to the microgametophytes at different stages in their lifecycle (during microsporogenesis, post-anthesis, and during pollen tube growth). Reciprocal experiments were performed and the progeny generations of the control and selected microgametophytes were compared. Pollen aging produced a progeny population with a greater mean plant size and reduced coefficient of variation then that of the control progeny. High temperature (29.5°C) pollen selection increased progeny height and leaf number. However, air drying and low temperature (15°C) pollen selection decreased mean plant size and/or branch number and increased the coefficient of variation. Results suggest that microgametopytic selection can be effective in shifting the mean of the progeny generation; however, the results obtained vary with selective agent, timing of selection, and the genotype of the parents used.

This thesis is dedicated to the memory of my grandparents...

Haskell and Olga Cunningham

Homer and Esther Mulinix

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KEY TO ABBREVIATIONS

CV - Coefficient of Variability

CDM - 15°C (Cold) During Microsporogenesis

CDPTG - 15°C (Cold) During Pollen Tube Growth

FDR - First Division Restitution

HDM - 30°C (Heat) During Microsporogenesis

HDPTG - 30°C (Heat) During Pollen Tube Growth

MDH - Malate Dehydrogenase

P - (Purple Alfalfa Clone) 299049 Plant 1

P x Y - Hybrid progeny; P maternal parent

Prx - Peroxidase

SDR - Second Division Restitution

Y - (Yellow Alfalfa Clone) W71-42 Plant 2

Y x P - Hybrid progeny; Y maternal parent

INTRODUCTION

The time required for sour cherry (Prunus cerasus L.) seedlings to reach fruiting is greater than for many crops. Hence, plant breeding programs involving this tree species, or others, must contribute a large proportion of resources to plant maintenance. In this study, gametophytic selection was investigated as a possible means of improving the efficiency of sour cherry and similar breeding programs.

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. In slowly maturing crops, microgametophytic selection could prove particularly advantageous because if successful: (1) fewer undesirable seedlings would be grown, (2) the probability of obtaining superior seedlings would be increased, and (3) the mean value of the seedling population would be shifted for the character(s) undergoing selection pressure.

It was impractical to use sour cherry for this study because of its long generation time and the difficulties associated with obtaining hybrid progeny. Therefore, alfalfa (Medicago sativa L.) was chosen, because like sour cherry, it is an outbreeding tetraploid (2N=4X) and may have the potential for microgametophytic heterosis. Furthermore, alfalfa possesses a short generation time and is easily propagated by stem cuttings.

The three major objectives of this study were: 1) to determine if selection at the gametophytic level could shift the average character values (height, leaf number, dry weight, etc.) of the resulting progeny generation, 2) to compare selection pressures applied at different stages of the microgametophyte's lifecycle (during microsporogenesis, post-anthesis, and during pollen tube growth), and 3) to distinguish what specific gametophytic selection pressures (freezing, air drying, aging, and high and low temperature) result in beneficial shifts in the sporophyte population.

LITERATURE REVIEW

MENDELIAN TRAITS EXPRESSED IN THE MICROGAMETOPHYTE

Numerous examples of simply inherited pollen expressed genes are present in the literature. The most frequently studied of these Mendelian inherited microgametophytic genes are the "S" alleles which govern incompatability reactions (Brewbaker, 1957; Brewbaker, 1959; Linskens, 1964; Townsend, 1971). Other widely studied microgametophytic genes include: gametic factor (Ga) which influences the pollen tube growth rate (Mangelsdorf and Jones, 1926), alcohol dehydrogenase locus (Adh) which codes for the Adh enzyme (Schwartz and Osterman, 1976), and the waxy (wx), sugary (Su), and shrunken (Sh) loci of maize (Zea mays L.) which alter the pollen's carbohydrate and amino acid content (Pfahler and Linskens, 1970a & 1970b). Studies of these gametophytic genes illustrated that pollen phenotype is at least partially dependent on gametophytically expressed genes. More recently, studies have determined that the microgametophytic generation expresses a vast number of genes which correspond to genes expressed during the sporophytic generation.

DETECTING GAMETOPHYTICALLY TRANSCRIBED GENES

Mulcahy et al. (1979) developed an electrophoresis technique which was capable of resolving the proteins contained in a single pollen grain. Using this technique, it was demonstrated that segregation for two protein genes was occurring in the pollen of heterozygous Cucurbita plants. Later, Mulcahy et al. (1981) found that at least 30% of the acid phosphate loci of selected Cucurbita spp segregated for expression in the pollen grains. Thus, Mulcahy concluded that the gametophytic generation transcribes, translates, and expresses several genes.

Gametophytically transcribed genes which correspond to sporophytically transcribed genes were compared by Tanksley et al.(1981). In this study, tomato plants known to be heterozygous for nine dimeric enzymes were used as pollen sources. If transcription and translation of the dimeric enzymes occurred in the haploid gametophyte then only homodimers (not heterodimers) would be detected by pollen protein electrophoresis. It was determined that 60% of the genes coding for these enzymes in the sporophytic generation were also expressed by the gametophytic generation. Rajora and Zsuffa (1986) obtained simular results studying the gametophytic and sporophytic proteins from three species of Populus (poplar).

Another appoach to determining the genetic overlap

between the sporophytic and the gametophytic generation involved DNA hybridization. Willing and Mascarenhas (1984) hybridized Tradescantia paludosa pollen cDNA to shoot RNA and found that at least 64% of the mRNA sequences expressed by pollen were expressed by the shoot tissues. It was also estimated that approximately 20,000 and 30,000 genes are expressed in the pollen and vegetative shoots respectively. These studies provided evidence that microgametophytes express many genes and that a majority of these genes are identical to those transcribed and translated by the sporophyte.

IN VITRO POLLEN GERMINATION AND TUBE GROWTH

Numerous studies of pollen germination and tube growth in vitro have focused on optimizing the media for pollen growth in order to study pollen physiology and test pollen viability. In 1961, Johri and Vasseł published an extensive review of work reporting the effect of sugars, growth regulators, vitamins, boron, and other chemicals on in vitro pollen growth. Other review articles by Brewbaker (1959) and Linskens (1964) provide additional information on in vitro pollen culture.

As a result of in vitro pollen growth studies, pollen culture media for optimal germination and tube growth have been developed. A medium which gives favorable results in

the culture of pollen from many taxonomic groups was presented by Brewbaker and Kwack (1963). It contained among its ingredients sucrose, boron, and calcium which have been shown to enhance pollen germination and tube growth in vitro. In addition, specific media have been developed for the culture of alfalfa pollen (Barnes and Cleveland, 1963a; Barnes and Cleveland, 1972; Lehman and Puri, 1964; and Puri and Lehman , 1965), filbert pollen (Zielinski, 1968), grape pollen (Bamzai et al., 1967), jojoba pollen (Lee et al., 1985), lettuce pollen (Eenink et al., 1983), maize pollen (Pfahler et al., 1967), pea pollen (Layne et al., 1964; Warnock et al., 1956), potato pollen (Mortenson et al., 1964), tomato pollen (Mc Leod, 1975), and so on.

With optimized pollen germination and growth media, it became possible to study the effect of pollen genotype on in vitro performance. Simon and Peloquin (1976) compared germination of 2N potato (Solanum spp) pollen formed by either the meiotic process of first division restitution (FDR) or second division restitution (SDR). The more heterozygous FDR pollen had statistically higher in vitro germination percentages and viability when fresh and following storage than the less heterozygous SDR pollen. The FDR 2N pollen also had a lower decline in germination ability upon storage than the SDR 2N pollen. The researchers concluded that the gametophytic vigor observed in the FDR 2N potato pollen was

at least partially attributable to microgametophytic heterosis.

Further work on the relationship between *in vitro* pollen performance and pollen genotype has been done using maize. Pfahler (1970) compared the *in vitro* germination and tube growth of three maize (*Zea mays* L.) inbreds and two of their single cross hybrids. There were clear differences in the pollen tube length and rate of growth of the inbreds. Examination of the germination and tube growth of the hybrids led to the conclusion that inheritance of these *in vitro* pollen growth characteristics of maize is complex and is probably polygenic. Similarly, Sari Gorla et al. (1975) found that single cross hybrids of maize have greater variances in pollen tube length (after one hour of culture) than their inbred progenitors. These results are expected if genes influencing pollen tube length are segregating in the pollen of the hybrids.

Evidence for segregation of genes influencing pollen tube length *in vitro* has also been demonstrated in alfalfa (*Medicago spp*). Barnes and Cleveland (1963a, 1963b, & 1972) compared the pollen tube lengths among alfalfa clones and found that there were significant differences between the pollen tube lengths after 16 hours of culture. Most of the alfalfa clones had polymodal distributions of their pollen tube lengths *in vitro*. However, some clones produced pollen

which yielded nearly all long or all short tubes. These findings indicate that alfalfa pollen tube length is, to some extent, genetically determined.

GAMETOPHYTIC AND SPOROPHYTIC RESPONSES TO STRESS

The demonstration that a large number of genes present in the sporophyte are expressed in the gametophyte has led to studies comparing the gametophyte's and sporophyte's response to stress. A correlation between the gametophyte's and sporophyte's response to stress would suggest that gametophytic selection schemes could have potential for modifying the resulting sporophytic generation.

Temperature is a major stress commonly applied to both the gametophytic and sporophytic generation. Weinbaum et al. (1984), working with several almond (Prunus dulcis L.) and peach (Prunus persica L.) cultivars, found that percent pollen germination in vitro at low temperatures was not related to the the chilling requirements and/or bloom date of the almond or peach cultivars. However, Weinbaum et al. did find significant differences between the two Prunus species for percent pollen germination at low temperatures. These differences indicated that almond pollen had greater cold tolerance than peach pollen. Zamir et al. (1981 & 1982) and Zamir and Vallenjos (1983) also found differences between two related Lycopersicon species for gametophytic

low temperature tolerance. In these studies Lycopersicon hirsutum L., a cold tolerant tomato species, was found to have greater gametophytic cold tolerance than the cold sensitive cultivated tomato Lycopersicon esculentum L.. This was tested by comparing percent pollen germination at 5°C and by comparing the results obtained when plants were pollinated using pollen mixtures of the two species at low and moderate temperatures. On the other hand, Maisonneuve et al. (1984) found that within the species of Lycopersicon esculentum L., in vitro pollen growth at low temperatures and plant growth of the pollen parent at low temperatures was not correlated. Furthermore, Den Nijs et al. (1986) and Maissonneuve et al. (1986) found that leaf initiation rates and average dry weights of F_2 test populations failed to indicate differential pollen selection for low temperature tolerance during either pollen formation or function. However, the authors indicated that pollination date effects might have interfered with detection of pollen selection effects. They also indicated that selective pressures may not have been extreme enough and variability for cold tolerance in the parental material may have been too low to detect differences in the F_2.

In maize (Zea mays L.), genotypic differences were found among inbred lines for pollen's ability to germinate in vitro after being subjected to post-anthesis heat stress

(Herrero and Johnson 1980). The most heat tolerant pollen came from popular inbreds of the 1970's while the least heat tolerant pollen came from inbreds used commonly in the 1930's. It was suggested that heat tolerance in the microgametopyte resulted from genetic improvement of the inbreds.

Weaver et al. (1985), suggested that heat tolerant bean plants may be detected by pollen staining during periods of high temperature. In their study they found that pollen from heat tolerant species had greater apparent pollen viability than heat sensitive species (as noted through pollen staining techniques) when pollen parents were grown at high temperatures.

Other studies which have found correlations between the male gametophyte and sporophyte for traits other than temperature tolerance include: (1) the ability of pollen to germinate in the presence of a pathotoxin and sporophytic resistance to that pathotoxin (Laughnan and Gabay, 1973), (2) saline tolerance of pollen during fertilization and salt tolerance plus vigor in the progeny (Sacher et al., 1983), (3) metal tolerance of Silene dioisa L., S. alba Mill. and Mimulus guttatus DC. pollen and copper or zinc tolerance in the sporophyte (Searcy and Mulcahy, 1985a & b), (4) ability of pollen to survive exposure to allyl alcohol and lack of alcohol dehydrogenase in the pollen parent (Schwartz and Osterman, 1976), (5) ozone sensitivitity of tobacco pollen

and ozone sensitivity of the pollen source (Feder, 1968), (6) age of pollen and dry weight of progeny--older pollen producing heavier offspring (Mulcahy et. al., 1982), and (7) the freezer storage of freshly collected potato pollen with a reduction of berry weight and seeds per berry in the subsequent progeny generation (Pallais et al., 1986).

POLLEN COMPETITION STUDIES

Mulcahy (1979) has hypothesized that the Angiosperm's rise to dominance was assisted by naturally occurring micro-gametophytic selection. Intense competition between male gametophytes screened out inferior haploid genomes, raising the mean performance levels of the following sporophytic generation.

Several studies correlated microgametophytic competition level with vigor in the subsequent sporophytic population. In maize, a basipetal increase in fertilization frequency of one pollen type over another on an ear has been used as an indication of that pollen's greater competetive ability (Since styles reaching the basel kernals on an ear of corn are longer than styles reaching apical kernals). Mulcahy (1974) reported a significant correlation between pollen tube growth rate of corn and the weight of the resultant seedlings after the factor of seed weight was removed. In his study, Mulcahy compared the pollen of

inbred lines and divided the lines into pairs of faster and slower pollen growth types. Then he crossed these pairs of inbreds separately onto a third inbred. The central seeds from the cobs of the two hybrid crosses made were planted for comparisons. Besides finding higher seedling weights for the hybrids produced by the faster pollen growth type it was found that the resultant seedling's total kernel and ear weights were positively correlated to the paternal parent's pollen tube growth rate (Ottaviano et al., 1980).

Similar competition studies have been performed using Dianthus chinensis by pollinating on either the tip or base of the long stigma. Seeds resulting from tip pollinations (high competition) were found to germinate significantly quicker than seeds from base pollinations (low competition). The tip progeny also had higher seedling weights (Mulcahy and Mulcahy, 1975). The authors concluded that a heterogenous pollen population subjected to high competition levels may yield more vigorous progeny.

Pollen competition has also been studied by varying the number of pollen tubes growing in the style. Mulcahy et al. (1975) performed "dot", "half", and "full" pollinations on the stigmas of a petunia plant (Petunia hybrida). The average seedling weight resulting from the "half" and "dot" treatments were 95% and 90% that of the "full" treatment. Thus, the full treatment appeared to yield the most vigorous offspring. On the other hand, in a similar experiment by

Ter-Avanesian et al.(1978) on Gossypium hirsutum (cotton), Triticum aestivum (wheat), and Vigna catjung (a bean species), the progeny means measured were not significantly different among the pollen treatments used to obtain the progeny. However, the variation of the metric character means of the sporophytes resulting from limited pollinations was greater than those of the sporophytes resulting from the excessive pollinations. Lewis (1954) reported similar results with tomato (Lycopersicon esculentum L.). These results suggest that pollen competition may serve to eliminate extreme pollen genotypes but may not, in some cases, change the mean performance of the resulting sporophytic generation.

MATERIALS AND METHODS

PLANT MATERIAL

Seed of two alfalfa accessions, PI 299049 and GP W71-42, was acquired from the North Central Regional Plant Intoduction Station at Ames, Iowa 50010 and from E. T. Bingham, Agronomy Department, University of Wisconsin, Madison, WI 53706, respectively. From the seedlings of these two populations, one plant per population was selected and increased by vegetative propagation. These plants were selected on the basis of flower color and apparent diversity from one another. The 299049 and W71-42 clones have purple (P) and yellow (Y) flower colors, respectively (Figure 1) which were used as a hybridization marker. Hybrid progeny between the P and Y clones had yellow/purple variegated flower color (Figure 1).

P.I. 299049, an alfalfa accession from Minnesota, was reclassified in 1978 under the name Medicago sativa L. subspecies sativa from the obsolete specis M. mesopotamica Vass. (Gunn et al., 1978). M. mesopotamica Vass., previously was specific for an unique, geographically isolated race of alfalfa that evolved in ancient Mesopotamia (Sinskaya, 1950). Mesopotamian alfalfa was characterized as more

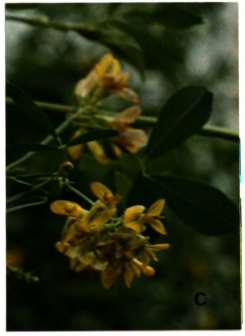

Figure 1: Alfalfa flower color: a. purple, b. yellow, c. variegated

vigorous, earlier maturing and less winter hardy than other races, and therefore more suitable for cultivation in mild environments. When M. mesopotamica Vass. spread to other areas and grew alongside hardier Central Asian alfalfa cultivars, new hybrids were formed. Plants of the former species M. mesopotamica Vass. were then classified into two ecotypes: the True Mesopotamian Ecotype and the Central Asiatic Hybrid Ecotype.

The purple flowering alfalfa clone chosen from the 299049 accession for these studies, P, does not conform to the True Mesopotamian Ecotype described by Vasil'chenko (1946). Vasil'chenko describes this True Mesopotamian Ecotype as having light green leaves and stems which are both covered with a heavy white pubescence. P, although possessing a moderately pubescent exterior, has dark green leaves and stems. P is also a long day plant while Mesopotamian alfalfa is day neutral. Furthermore, Vasil'chenko describes M. mesopotamica Vass. as tall (70-80cm) with long internodes (4.6-5.6cm). P was found to be shorter and more compact than this in structure when grown in large pots in the greenhouse (Table 1; Figure 2). Therefore, it is apparent that P is not of the True Mesopotamican Ecotype and must be of hybrid origin. Nevertheless, P does have several characteristics in common with Mesopotamian alfalfa. These characteristics include: 1) an upright growth habit, 2) a thick taproot with few lateral roots, 3) short branches, 4) purple flowers, 5) coiled legumes, 6) a tetraploid 2N=4x=32

chromosome number, 7) fast regrowth after cutting, and 8) early flowering. It may also possess the following features of Mesopotamian alfalfa: 1) high heat and drying wind resistance, 2) low cold tolerance, 3) low drought tolerance, 4) low winter survival (P.I.- 299049 had 56% winter survival at Ames, Iowa (Gunn et al.,1978)), and 5) high disease susceptibility.

The alfalfa synthetic GP W71-42, released in 1975, was described as consisting of "mainly hybrids between cultivated alfalfa Medicago sativa L. and wild diploid M. falcata L." (Bingham, 1975). This statement is no longer accurate since the former species M. falcata L. is now considered M. sativa subspecies falcata.

Clone W71-42-plant 2 or Y, according to the key presented by Gunn et al. (1978), is classified as M. sativa subspecies falcata which characteristically has yellow flowers and noncoiled legumes. If it was a hybrid (as Bingham described) between a M. sativa subspecies sativa (coiled pods, purple flowers) and a M. sativa subspecies falcata (falcate pods, pure yellow flowers), one would expect an intermediate phenotype with weakly spiraled legumes and variagated flowers. This intermediate phenotype would be of the subspecies x varia. However, Y does not fit this description, but fits the description of the falcata subspecies (Table 1; Figure 2).

Plants of the falcata subspecies of alfalfa are found growing naturally across the U.S.S.R. (Gunn et. al., 1978).

There is a wide variety of ecotypes of this subspecies with one of the hardiest ecotypes found growing naturally near the arctic circle. For simplicity, Y will be compared with a composite of the traits common to these various falcata ecotypes.

Y, although tetraploid, has many characteristics in common with the typical diploid M. sativa subspecies falcata. These characteristics are: 1) a strong and branching root system, 2) numerous stems arising from the crown, 3) falcate shaped pods, 4) slow regrowth after cutting (Y is only somewhat slower than P), and 5) a long-@day photoperiod. It also may share the following characteristics with M. sativa subspecies falcata: 1) poor heat tolerance, 2) cold tolerance, 3) drought resistance, 4) high winter survival (A majority of the accessions used to construct GP-W71-42 had 100% winter survival at Ames, Iowa (Gunn et al., 1978)), and 5) low disease susceptibility.

Table 1: Comparisons of the P and Y Plant Material

P	versus	Y
M. sativa subsp. sativa		M. sativa subsp. falcata
Formally M. mesopotamia		Formally M. falcata
Origin-Mesopotamia (Iraq)		Origin-(Probabily) Russia
Purple Flowers		Yellow Flowers
Earlier Maturing		Later Maturing
Faster Growing		Slower Growing
Shorter		Taller
More Branches		Fewer Branches
More Leaves		Fewer Leaves
More Apparant Heat Tolerance		More Apparent Cold Tolerance

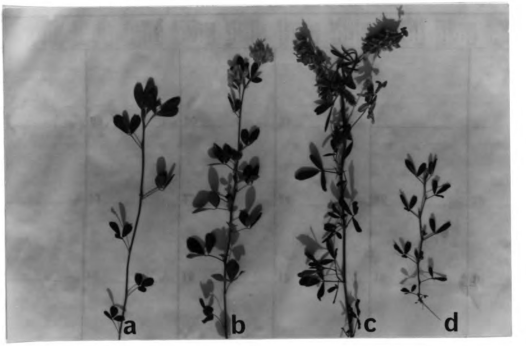

Figure 2: Plant material: a. vegetative Y, b. flowering Y, c. flowering P, d. vegetative P

POLLEN TREATMENTS AND POLLINATION PROCEDURE

I. Selection During Microsporogenesis

a.& b. 29.5°C (High) and 15°C (Low) Temperature

Treatments-One set of the Y and P alfalfa genotypes was placed into a 29.5°C growth chamber (Feb. 15, 1985) and another set was placed into a 15°C growth chamber (March 8, 1985) under 16 hour cool-white flourescent and incandescent bulbs (~325 μmol $s^{-1}m^{-2}$). Relative humidity in the growth chambers was not controlled. Three weeks after the plants were placed in the growth chambers, they were moved back into a 22°C greenhouse (natural photoperiod being supplemented with indirect 16 hour high-pressure sodium lighting). Freshly shed pollen from 120 to 125 flowers of the Y and P clones which had undergone microsporogenesis in either the 29.5°C growth chamber, 15°C growth chamber or in the 22°C control greenhouse environment, was gathered. This pollen was used to make hybrid crosses onto the emasculated flowers of an additional greenhouse grown clone of Y and P. Cross pollinations were made as indicated in Table 2. Upon completion of the pollinations the plants were grown in a 22°C/16 hour photoperiod greenhouse till May, then in a 18°C/16 hour photoperiod greenhouse. Seed was collected when the pods turned brown and dry on several occasions from April 12-May 13, 1985.

Table 2: Pollination and seed set data of crosses

Cross Pollination	No. of flowers Pollinated	% Pod Set	Mean No. of Seeds/Pod[y]
I.a. 29.5°C (Heat) During Microsporogenesis Experiment			
P x Y-Control	108	62	4.64
P x Y-Heat	110	47	4.71
Y x P-Control	57	26	4.07
Y x P-Heat	67	51	4.91
I.b. 15°C (Cold) During Microsporogenesis Experiment			
P x Y-Control	95	15	2.07
P x Y-Cold	95	20	1.74
Y x P-Control	75	52	4.95
Y x P-Cold	78	59	4.04
II.a. Mature Pollen Freezing (42 Days) Experiment			
P x Y-Control	30	17	2.00
P x Y-Frozen	32	44	3.36
Y x P-Control	30	40	5.00
Y x P-Frozen	37	57	4.19
II.b. Mature Pollen Aging (7 Days) Experiment			
P x Y-Control	48	60	1.66
P x Y-Aged	32	50	2.00
Y x P-Control	46	67	1.48
Y x P-Aged	136	24	3.97
III.a. 29.5°C (Heat) During Pollen Tube Growth Experiment			
P x Y-Control*	85	36	3.84
P x Y-Heat	85	68	3.52
Y x P-Control	107	26	5.33
Y x P-Heat	104	51	5.70
III.b. 15°C (Cold) During Pollen Tube Growth Experiment			
P x Y-Control*	100	49	2.94
P x Y-Cold	100	49	3.31
Y x P-Control	74	52	4.15
Y x P-Cold	75	59	4.60

[y]Excluding aborted seeds

*Control temperature 22°C

II. Selection Following Anthesis

a. Pollen Freezer Storage (42 Days) Treatment- On February 11, 1985 pollen was collected from 100 flowers of the greenhouse grown (22°C/16 hour photoperiod) P and Y alfalfa clones. Cross pollinations were immediately made using a portion of this pollen (Table 2). The two dram vials containing the remainder of the pollen were then placed in the freezer at -16°C for 42 days (Pollen was allowed to reach room temperature once for ~1.5 hours on March 11th). On March 25th crosses were repeated onto the same set of plants using the pollen which had been frozen. Seed was collected on March 28 and May 3.

b. Pollen Aging (7 Days) Treatment- On June 20, 1985 pollen was gathered from 200 flowers each of the greenhouse grown (18°C/16 hour photoperiod) P and Y alfalfa clones. At that time, crosses were made onto greenhouse plants using a portion of the collected pollen (Table 2). The remaining pollen was placed in 2 dram capped vials wrapped with Parafilm® and foil, and stored at 25°C for 7 days. Crosses were repeated using the 7 day old pollen with the same set of plants. Seed was collected on July 22 and 29.

c. Pollen Air Drying (24 Hours) Treatment- Y x P-dried crosses were performed on two spring dates. The pollen used in these Y x P crosses was lightly layered in a clear glass vial and left uncapped at ~25°C for approximately 24 hours before being used in the pollinations.

Control Y x P crosses were made on five dates in the spring of 1984. The P pollen used was collected just prior the the pollination procedure. Seed was collected from these crosses on April 11 & 23, and May 29.

No P x Y crosses were made

III. Selection During Pollen Tube Growth

a.& b. 29.5°C (High) and 15°C (Low) Temperature Treatments- On March 5 and March 20, 1985 pollen was collected from approximately 200 flowers of both the greenhouse grown Y and P alfalfa clones. Following pollen collection on these two dates, hybrid crosses were made in duplicate on two P and two Y clones. One plant of each pair was kept in the 22°C greenhouse with 16 hour photoperiod (lighting previously described) and the other was placed (within two hours after the first crosses were made) into a 29.5°C (3-5-85) or a 15°C (3-20-85) growth chamber with 16 hour lighting (lighting also previously described). Four days later, pod formation was evident and the plants in the growth chamber were placed next to the others in the 22°C/16 hour photoperiod greenhouse. Later they were moved to a 18°C/16 hour photoperiod greenhouse and seed was collected on several dates from April 1 to May 16.

POLLEN GERMINATION

In vitro pollen germination tests were done on subsamples of the Y and P pollen used in the hybridizations (except for the air drying experiment). Pollen was cultured for one hour in 3.5 cm petri plates containing 1 ml of pH 6.6 Brewbaker's medium (Brewbaker and Kwack, 1963). Pollen was germinated at three different temperatures: 15°C, 22°C, and 30°C. Furthermore, tests were done before and after the aging and freezing stresses. At the end of the culture period the petri plates were placed in a 4°C refrigerator to retard further pollen tube growth. Germination counts were started an hour after the petri plates were placed into a refrigerator (4°C). A total of 125 swollen grains per plate were scored as germinated or nongerminated depending on the presence or absence of a pollen tube, respectively.

Several tests were performed to determine the effect of air drying on *in vitro* pollen germination, but these tests did not include a subsample of the pollen used for the Y x P hybridizations and were done in pH 7.0 Brewbaker's medium.

SEED GERMINATION AND SEEDLING GROWTH

Seeds from five of the seven experiments (exceptions mentioned below) were sown on June 12, 1985 in 72-cell plastic flats filled with soil mix (Baccto®). The seeds were pressed into the soil surface at the rate of one seed per cell. Flats were placed in a 22°C greenhouse under natural light.

When the alfalfa seedlings emerged, the germination date for each was recorded. Seedlings with their first trifoliate leaf, were transplanted into 1.5" (3.81 cm) diameter X 8.5" (20.32 cm) tall "cone-tainers®" (Ray Leach Cone-Tainer® Nursury, 1787 N. Pine St., Canby, Oregon 97013. Ninety-eight cone-tainers® were situated in each 1' X 2' (30.5 cm X 61.0 cm) cone-tainer® rack). The seedlings were fertilized weekly (Peters® 20-20-20). Flowering date and flower color of the seedlings was recorded.

Seeds of the pollen aging (7 Days) experiment were sown in large clay pots filled with soil mix (Baccto®) on August 5, 1985. The resulting seedlings were transplanted into cone-tainers® as previously described.

Seeds of the pollen air drying (24 Hours) treatment were sown in rows in flats filled with soil mix (Baccto®) on June 18, 1984. Seedlings were transplanted into 4" clay pots.

CUTTINGS AND GROWTH CHAMBER EXPERIMENTS

I. Selection During Microsporogenesis

a. 29.5°C (High) Temperature Treatment- On January 2-3, 1986, three cuttings per plant were taken from the actively growing greenhouse stock plants to minimize the confounding maternal effects of seed size and germination date on plant size (27°C Day/21°C Night, natural light and supplemental 16 hour photoperiodic lighting). A few weeks prior to the process of taking the cuttings the flowering plants were cut back several inches so new vegatative growth would be initiated. The cuttings consisted of single two-node segments from directly below the apical meristem of separate stems. These segments all lacked lateral branching. If it was not possible to get three cuttings per plant, fewer then three cuttings were taken. The bottom leaf of each two leaf cutting was removed and the cuttings were dipped into a rooting powder (Rootone®) and placed into a cell filled with 1:1 soil mix and perlite (72 cells per flat; 3 cuttings of one genotype per cell). The cuttings were placed under intermittent mist until January 12th.

On January 12, 1986 the one best rooted cutting of the three taken from each seedling was transplanted into a soil filled cone-tainer®. The bottom node of the cuttings was completely buried during transplanting. Since there was insufficient space in the growth chamber for all the

Figure 3: a. Segment selected for cuttings, b. cuttings ready for rooting

cuttings, plants were randomly eliminated from groups with excessive individuals. The plants were arranged in a completely randomized design and placed in a 30±4°C walk-in growth chamber under 16 hour cool-white flourescent and incadescent light. This temperature was believed to be suboptimal for alfalfa growth making progeny differences easier to detect. The plants were watered daily and fertilized with Peters® 17-17-17 on January 17, 24 and February 7.

On February 13th, the cuttings were removed from the growth chamber and plant height was recorded to .5 cm. Following this the plants were cut off at the soil level and the top green matter portion was saved for further analysis. The root material was rated on a scale from 1-4 with 1 being minimal root growth and 4 being extremely heavy growth. After being rated the root portions were discarded. The green matter sections, meanwhile, were oven dried at 60°C for over a week. The cuttings were evaluated for: branch number, leaf number, total leaf dry weight (to .01g), and total green matter dry weight (to .01g).

Descriptions of the various cutting characters are presented below.

BRANCH NUMBER-A branch was considered to be a stem or node which contained three or more leaves (young plants have 1 leaf per node). The main stem was considered to be a branch.

HEIGHT-Height was the distance from the soil to the apical meristem of the longest stem. If the main stem was curved or twisted it was straightened as much as possible while measurements were taken.

LEAF NUMBER- All expanded trifoliate leaves were counted.

In the subsequent statistical analysis, the cuttings having a final rooting value of 1 were omitted. A very low number of cuttings were found with this rating and they grew rather poorly.

b. 15°C (Low) Temperature Treatment- Cuttings were taken from the Y x P progeny of the cold during microsporogenesis experiment on November 13, 1985. These cuttings, unlike the cuttings in the preceding experiment, were three nodes in length. Otherwise, they were treated similarly. From November 13 to December 3 the cuttings were kept under intermittent mist. After removal from the intermittent mist, the cuttings were transplanted and cut back to 1 node above the soil (to make them two nodes in length). On the same day they were placed into a 14°C growth chamber under 16 hour flourescent and incadescent lighting. On December 3, 13, 23 and January 3 the cuttings were fertilized with Peters® 20-20-20. The cuttings were removed from the growth chamber on January 13, 1986. Data was taken as previously described with the exception that total leaf dry weight was not measured and lowest stem width to .00254 cm was measured.

II. Selection Following Anthesis

a. Pollen Freezer Storage (42 Days) Treatment- Cuttings were taken on February 3, 1986 following the procedure described in the high temperature during microsporogenesis experiment and were rooted under intermittent mist until February 19. On February 22 they were transplanted into cone-tainers® and placed in a 14±4°C/16 hour cool-white fluorescent and incandescent lighted growth chamber with the cuttings from the pollen aging experiment and the 15°C (cold) during pollen tube growth experiment. These cuttings were fertilized with Peters® 17-17-17 on March 3, 10, 17, and 25. They were taken out of the chamber for data collection on March 29-31.

b. Pollen Aging (7 Days) Treatment- Cuttings were taken on February 4-5, 1986 and handled in the same manner as those from the pollen freezing experiment (Section II.a.).

c. Pollen Air Drying (24 Hours) Treatment- On January 26, 1985, ten cuttings per plant were taken from 45 randomly chosen Y x P-control plants and all 42 of the Y x P-air dried progeny. These three-node cuttings did not contain the apical meristem and were unbranched. It was impossible to take all the cuttings from the same age growth and some cuttings had to be taken from older, lower stem portions. After removing the bottom leaf, each cutting was dipped into

rooting talc (Rootone®) and placed into a soil filled cone-tainer® Racks of filled cone-tainers® were placed in large, clear plastic bags to create a humid environment for rooting. Once rooted, eight of the most uniform cuttings per plant were randomly divided into two sets of four. The cuttings in the two resulting groups were randomly arranged in the cone-tainer® racks. These racks were then placed into separate greenhouses under natural light (Greenhouse I=22°C, Greenhouse II=17°C). Data on plant height, branch number, and width of lowest branch were taken shortly before the plants were harvested to obtain green matter dry weights. Plants from greenhouse I were harvested on 4-3-85 (67 days after cuttings were taken), and plants from greenhouse II were harvested on 4-14-85 (78 days after cutting were taken). Roots of these plants were discarded without being evaluated. Measurements of the four cuttings per plant per greenhouse were averaged together to give one mean value.

III. Selection During Pollen Tube Growth

a. 29.5°C (High) Temperature Treatment- Cuttings were made on December 17-19, 1985 in the same manner as those for Section I.a. and placed under intermittent mist until January 3, 1986. During the wait for a growth chamber the transplanted cuttings were kept in a 27°C Day/21°C Night greenhouse with a 16 hour photoperiod from indirect lighting. Since growth chamber space was limited, several

individuals were randomly eliminated from the P x Y-Heat During Pollen Tube Growth (HDPTG), Y x P-Control, and Y x P-HDPTG groups. On January 12, the remaining plants were cut back to two above ground nodes and placed in a completely randomized design in the 30±4°C growth chamber under full 16 hour cool-white flourescent and incadescent lighting. Plants were watered once or twice daily and fertilized with Peters® 17-17-17 on January 17, 24, and February 17. Several cuttings suffered from fertilizer burn due when the soil dried out after the second fertilization and were removed . The rest were rearranged to fill in the empty spots in the cone-tainer® racks. Data on plant height were taken on the plants on either February 16 or 17. Further data were collected as described in section I.a..

b. 15°C (Low) Temperature Treatment- Cuttings were made on February 4-5, 1985 and treated similar to those in the pollen freezing experiment (Section II.a.).

RESULTS

SEED AND POD SET

Percent pod and seed set varied among the cross pollinations performed (Table 2). In some cases, the actual pod set may differ from the value given in Table 2. During a move from one greenhouse to another, pods and labels were ripped from a few of the alfalfa plants . Furthermore, the suction emasculation procedure used for the cross pollinations undoubtedly damaged some of the styles resulting in a reduced pod set. Additional information on pod and seed set from two of the experiments is discussed below.

Mature Pollen Aging (7 Days) Treatment- In this experiment, all the pollinations made on June 20 yielded a large percentage of "aborted" seeds. These small shrunken seeds, which were not included in the seed set data, represented 24% of the P x Y-Control seeds, and 62% of the Y x P-Control seeds. The other experiments seldom had more than 5% aborted seeds for any group.

The mean number of seeds per pod of the Y x P-Aged

group was significantly higher according to t-test than mean seed number per pod of the Y x P-Control group ($P < .05$). This apparently reflects the high number of aborted seeds in the control cross.

Mature Pollen Air Drying (24 Hours) Treatment- Data on pod set and seed set was not taken. A subsequent attempt to repeat the experiment failed due to a very low pod set of all the crosses involved.

POLLEN GERMINATION *IN VITRO*

29.5°C (Heat) During Microsporogenesis Treatment- No significant differences in percent pollen germination were found for pollen formed at control (22°C) and high (29.5°C) temperatures (Table 3). However, Y pollen which underwent microsporogenesis in the growth chamber at 29.5°C had abnormal growth with twisted and spiraling pollen tubes.

15°C (Cold) During Microsporogenesis Treatment- The P pollen which developed at 15°C had a high frequency of empty pollen grains (38.4%)(Table 3). These grains did not swell when placed in the germination media and were not counted in determining percent germination.

Mature Pollen Freezing (42 Days) Treatment- Freezer storage (1.5 months at -16°C) caused a significant decline in the percentage of Y pollen that germinated *in vitro* (80.5% to

TABLE 3: *In vitro* percent pollen germination for the P and Y alfalfa pollen at the time of pollination

I. Selection During Microsporogenesis

a. Heat

	POLLEN DEVELOPMENT TEMPERATURE	
	22°C	29.5°C
P	25.9[w] (6.9)a[x]	40.5 (13.6)a
Y	80.5 (8.8)a	70.1 (16.5)a

b. Cold

	22°C	15.0°C
P	58.7 (5.3)a	43.3 (6.1)a
Y	42.1 (3.3)a	45.9 (3.7)a

II. Selection After Anthesis

a. Pollen Freezing (-16°C)

	DAYS OF STORAGE	
	0	42
P	37.6 (10.4)a	22.7 (1.2)a
Y	80.5 (5.9)b	24.8 (3.7)a

b. Pollen Aging

	0	7
P	55.2 (10.0)b	14.9 (7.6)a
Y	83.2 (7.3)b	53.6 (6.4)a

III. Selection During Pollen Tube Growth

a. Heat

	CULTURE TEMPERATURE[z]	
	22°C	29.5°C
P	78.4 (2.8)a	61.9 (11.6)a
Y	62.7 (3.9)a	52.5 (6.1)a

b. Cold

	22°C	15.0°C
P	15.7 (11.2)a	18.0 (6.4)a
Y	25.1 (1.7)a	27.7 (6.5)a

( )Standard Deviation

[w]Means in percent based upon three samples of n=125

[x]Means indicated by the same letter within clone within experiment are not significantly different according to t-test (α=.05) of transformed data (arcsin[percentage]$^{1/2}$ transformation)

[z]Initial culture temperature was 22°C for all samples. At start of the one hour culture period samples were placed in the environment indicated.

24.8%). The decline observed for the P pollen was not significant.

Mature Pollen Aging (7 Days) Treatment- Aged pollen suffered a significant reduction in *in vitro* pollen germination. Mean P pollen germination dropped from 55.2% to 14.9% (a 73% loss of viablity) and Y pollen germination dropped from 83.2% to 53.6% (a 36% loss of viablitity)(Table 3).

Mature Pollen Air Drying (24 Hours) Treatment-Pollen germination tests were not performed on subsamples of the pollen used. Several tests were performed, however, to observe the effect of air drying on P and Y pollen germination *in vitro*.

P pollen which was dried for 24 hours, had *in vitro* germination percentages which ranged from 23 to 66% with an overall mean germination percentage of 38%. Fresh P pollen, collected the day following the pollen which was allowed to dry, had germination percentages which ranged from 42 to 92% with an overall mean germination percentage of 73%. Thus, the dried P pollen sample had about half the viability of the fresh P pollen when cultured for one hour at room temperature.

Y pollen allowed to air dry in a vial on the lab bench top had *in vitro* germination percentages which ranged from 44-70% with an overall mean of 57%. Freshly collected Y pollen had *in vitro* germination percentages which ranged from 78 to 82% with an overall mean germination percentage

of 80%. The in vitro germination of the air dried Y pollen samples was about 70% that of the fresh.

29.5°C (Heat) During Pollen Tube Growth Treatment-Pollen germination at the two temperatures did not differ significantly within maternal parent (Table 3). However, the pollen samples tested at the higher temperature initially began their culture as a 22°C pollen/medium suspension that was placed in the 29.5°C growth chamber. Since pollen germination of alfalfa is a rapid process, essentially complete after 30 minutes, these two groups were probabaly not subjected to widely differing temperatures during initial pollen germination. These groups were, however, subjected to different temperature environments during a large part of the tube elongation process. The P pollen samples cultured in the 29.5°C growth chamber had many tubes which were much longer than the tubes of the P pollen samples cultured in the greenhouse at 22°C. Y pollen samples cultured in the 29.5°C growth chamber, on the other hand, had a larger porportion of shorter length pollen tubes than the greenhouse cultured samples.

15°C (Cold) During Pollen Tube Growth Treatment-All pollen germination levels were low, perhaps due to prior pesticide application given the pollen parents (Table 3).

SEEDLING RESULTS

The progeny of cross pollinations were identified by variegated flowers which were a purple/green color when young and yellowed upon aging. Usually, mature Y x P and P x Y flowers were tannish yellow with purple veins. Occasionally, the flowers appeared to be green due to a combination of the purple and yellow pigments.

In some of the experiments there were insufficient numbers of P x Y-Control and Treatment seedlings to perform progeny tests. The P parent usually had lower pod set and yielded fewer seeds per pod than the Y parent.

SEED GERMINATION

29.5°C (Heat) During Microsporogenesis (HDM) Treatment- Seed of the Y x P-Control pollinations had 84% germination while seed of the Y x P-HDM pollinations exibited only 29% germination (Table 4). Seed from these treatments came from the same plant and from pollinations performed on the same day. Germination rates also varied between the seeds from the control and HDM crosses within maternal parent. The seeds of the P x Y-HDM treatment germinated on the average of 10.8 days earlier than the P x Y-control seeds. The

TABLE 4: Seed germination of the progeny resulting from the 29.5°C (heat) during microsporogenesis experiment

CROSS POLLINATION	PERCENT SEED GERMINATION	MEAN DAYS FROM SOWING TO SEED GERMINATION
P x Y-control	79.4	37.3 b*
P x Y- 29.5°C (heat) during microsporogenesis	83.3	26.5 a
Y x P-control	83.6	45.8 a
Y x P- 29.5°C (heat) during microsporogenesis	28.7	61.3 b

*Means indicated by the same letter within one pair are not significantly different according to t-test (α=.05)

( )Coefficient of Variation

Y x P-HDM seeds, on the other hand, germinated on the average 15.5 days later than the Y x P-control seeds.

Mature Pollen Aging (7 Days) Treatment-Seed germination percentages were similar for all the groups (~70%).

29.5°C (Heat) During Pollen Tube Growth (HDPTG) Treatment-Seed germination was high for all the treatment groups (>67%). The P x Y-HDPTG seeds germinated very rapidly and much less sporadically than seeds from other experiments (data not presented). These seeds also had a larger mean seed weight than most groups of seeds from the P clone.

15°C (Cold) During Pollen Tube Growth (CDPTG) Treatment-Seed germination was higher within maternal parent for the CDPTG groups (P x Y, 89%; Y x P, 73%), than the Control groups (P x Y, 72%; Y x P, 63%).

CUTTING CHARACTER MEASUREMENTS

29.5°C (Heat) During Microsporogenesis (HDM) Treatment-Plant height was the only character measured which differed significantly between the control and HDM cross pollinations after 32 days in a 30±4°C growth chamber (Table 5). The mean height of the rooted cuttings of the P x Y-HDM offspring was larger than that for the P x Y-control progeny. There were no differences between the control and HDM progeny treatment in the reciprocal cross.

Table 5. Mean plant height, number of branches, number of leaves, total leaf dry weight, and total green matter dry weight of the progeny resulting from the high temperature during microsporogenesis experiment when rooted cuttings were grown for 32 days at 30°C.

Cross pollination - treatment	Number of progeny	Plant height (cm)	Number of branches per plant	Number of leaves per plant	Total leaf dry weight per plant (g)	Total green matter dry weight per plant (g)
P x Y - Control	77	32.8 (22)a[z]	7.1 (48)a	35.7 (37)a	.170 (37)a	.310 (40)a
P x Y - Heat during Microsporogenesis	74	35.2 (20)b	7.0 (47)a	32.9 (35)a	.170 (34)a	.327 (35)a
Y x P - Control	49	38.5 (19)a	6.7 (44)a	34.6 (34)a	.165 (36)a	.336 (40)a
Y x P - Heat during microsporogenesis	43	38.1 (26)a	6.7 (72)a	33.3 (55)a	.146 (48)a	.299 (49)a

[z]Means indicated by the same letter within one pair are not significantly different according to T-test ($\alpha = .05$).

( ) Coefficient of variation.

Although the means of the other characters measured did not differ significantly between the P x Y-control and P x Y-HDM cuttings, in most cases the P x Y-HDM cutting characters had lower coefficients of variability (CV's). Y x P-HDM cuttings, in contrast, had higher CV's for the characters measured than the Y x P-control cuttings.

15°C (Cold) During Microsporogenesis (CDM) Treatment-There were no significant differences between the plant character means of the Y x P progeny (Table 6). The CV's of the Y x P-CDM group were lower than the CV's of the control Y x P group for all characters measured.

P x Y cuttings were not availuable in sufficent numbers for comparison.

Mature Pollen Freezing (42 Days) Treatment-There were no significant differences between the character means between the Y x P-Control and Y x P-Frozen progeny (Table 7). The Y x P-Frozen progeny had slightly higher CV values than the Y x P-Control group for all characters measured.

P x Y cuttings were not availuable in sufficent numbers for comparison.

Mature Pollen Aging (7 Days) Treatment-The character means of the P x Y-Control and P x Y-Aged progeny did not differ significantly when rooted cuttings were grown for 5 weeks at 14°C (Table 8). However, the P x Y-Aged group consisted of only 14 individuals. For all measured characters, the P x Y-Aged progeny had slightly lower means with higher CV's than the P x Y-Control group.

Table 6. Mean plant height, number of branches, number of leaves, lowest branch diameter, and total green matter dry weight of the progeny resulting from the low temperature during microsporogenesis experiment when rooted cuttings were grown for 6 weeks at 14°C.

Cross pollination - treatment	Number of progeny	Plant height (cm)	Number of branches per plant	Number of leaves per plant	Diameter of lowest branch (cm)	Total green matter dry weight per plant (g)
Y x P - Control	48	28.4 (26)a[z]	2.2 (70)a	14.2 (47)a	.081 (31)a	.243 (61)a
Y x P - Cold during microsporogenesis	50	29.3 (22)a	2.4 (58)a	15.8 (38)a	.084 (27)a	.257 (50)a

[z]Means indicated by the same letter within one pair are not significantly different according to T-test ($\alpha = .05$).

( ) Coefficient of variation.

Table 7. Mean plant height, number of branches, number of leaves, total leaf dry weight, and total green matter dry weight of the progeny resulting from the post anthesis pollen freezer storage experiment when rooted cuttings were grown for 5 weeks at 14°C.

Cross pollination - treatment	Number of progeny	Plant height (cm)	Number of branches per plant	Number of leaves per plant	Total leaf dry weight per plant (g)	Total green matter dry weight per plant (g)
Y x P - Control	36-37	34.1 (15)a[Z]	5.2 (44)a	26.5 (31)a	.192 (32)a	.398 (35)a
Y x P - Frozen	49-50	34.6 (16)a	4.9 (49)a	25.9 (33)a	.186 (36)a	.377 (36)a

[Z]Means indicated by the same letter within one pair are not significantly different according to T-test ($\alpha = .05$).

( ) Coefficient of variation.

Table 8. Mean plant height, number of branches, number of leaves, total leaf dry weight, and total green matter dry weight of the progeny resulting from the post anthesis pollen aging experiment when rooted cuttings were grown for 5 weeks at 14°C.

Cross pollination - treatment	Number of progeny	Plant height (cm)	Number of branches per plant	Number of leaves per plant	Total leaf dry weight per plant (g)	Total green matter dry weight per plant (g)
P x Y - Control	30	31.1 (23)a[z]	4.8 (49)a	25.1 (36)a	.162 (49)a	.325 (51)a
P x Y - Aged	14	29.7 (35)a	4.2 (51)a	22.0 (50)a	.146 (55)a	.294 (61)a
Y x P - Control	50	32.5 (23)a	3.5 (58)a	19.1 (40)a	.151 (42)a	.308 (45)a
Y x P - Aged	70	34.0 (16)a	4.5 (54)b	22.7 (37)b	.185 (37)b	.376 (39)b

[z]Means indicated by the same letter within one pair are not significantly different according to T-test ($\alpha = .05$).

( ) Coefficient of variation.

The Y x P-Aged progeny differed significantly from the Y x P-Control with a greater branch number, leaf number, total leaf dry weight, and total greenmatter dry weight. In addition, the CV's for the Y x P-Aged progeny characters were consistently lower than those of the Y x P-Control progeny.

Pollen Air Drying (24 Hours) Treatment-The Y x P-Control cuttings grown for 67 days in a 22°C greenhouse/natural days differed significantly from the Y x P-Air Dried cuttings for all characters measured (Table 9). The control progeny population had a greater mean height, branch number, lower branch width, and total green matter dry weight than the Y x P-Air Dried progeny population. Cuttings of the same plants grown for 78 days in a 17°C greenhouse yielded the same results. For most of the characters measured the Y x P-Air Dried progeny had greater CV's than the Y x P-Control progeny. This was especially apparent for total green matter dry weight/plant.

There were no P x Y offspring to test.

29.5°C (Heat) During Pollen Tube Growth (HDPTG) Treatment-The Y x P-Control and Y x P-HDPTG cuttings grown for 5 weeks at 30°C did not differ significantly for any of the characters measured (Table 10). On the other hand, the P x Y-HDPTG progeny had significantly larger mean number of leaves per plant ($P < .05$) and just nonsignificantly greater mean plant height ($P = .056$) than the P x Y-Control progeny. In addition, all P x Y-HDPTG population character means

Table 9. Mean plant height, number of branches, lowest branch width, and total green matter dry weight of the progeny resulting from the post anthesis pollen drying experiment when rooted cuttings were grown either 67 days in a 22°C greenhouse or 78 days in a 17°C greenhouse.

Cross pollination - treatment	Number of progeny	Plant height (cm)	No. of branches per plant	Lowest branch width (cm)	Total green matter dry weight/plant (g)
Y x P - Control	45[y]	37.3 (18)b[z]	9.6 (35)b	.122 (8)b	.523 (21)b
Y x P - Dried	42	32.6 (25)a	7.7 (37)a	.111 (13)a	.401 (37)a
Y x P - Control	45[x]	56.3 (15)b	9.2 (34)b	.133 (13)b	.731 (23)b
Y x P - Dried	42	52.4 (18)a	7.8 (33)a	.124 (13)a	.577 (33)a

[z]Means indicated by the same letter within one pair are not significantly different according to T-test ($\alpha = .05$).

[y]Greenhouse temperature 22°C.

[x]Greenhouse temperature 17°C.

( ) Coefficient of variation.

Table 10. Mean plant height, number of branches, number of leaves, total leaf dry weight, and total green matter dry weight of the progeny resulting from the high temperature during pollen tube growth experiment when rooted cuttings were grown for 5 weeks at 30°C.

Cross pollination - treatment	Number of progeny	Plant height (cm)	Number of branches per plant	Number of leaves per plant	Total leaf dry weight per plant (g)	Total green matter dry weight per plant (g)
P x Y - Control	60	26.3 (23)a[z]	8.5 (44)a	41.1 (27)a	.156 (28)a	.280 (32)a
P x Y - heat during pollen tube growth	88	28.4 (24)a	9.1 (38)a	44.7 (25)b	.164 (23)a	.301 (26)a
Y x P - Control	97	24.6 (28)a	7.2 (32)a	37.2 (47)a	.173 (31)a	.294 (32)a
Y x P - heat during pollen tube growth	94	24.6 (32)a	6.8 (33)a	37.0 (49)a	.166 (31)a	.294 (35)a

[z]Means indicated by the same letter within one pair are not significantly different according to T-test ($\alpha = .05$).

( ) Coefficient of variation.

exceeded those of the P x Y-Control progeny and usually had lower CV values.

Note: Several of the cuttings began to flower in the growth chamber. Larger differences may have been found between cuttings if the data had been taken earlier.

15°C (Cold) During Pollen Tube Growth (CDPTG) Treatment- Population character means of the P x Y-Control versus the P x Y-CDPTG group were not significantly different (Table 11). The P x Y-CDPTG cuttings, however, were considerably more variable for dry weight measurements than the P x Y-Controls.

The Y x P-Control offspring had a significantly greater mean branch number than the Y x P-CDPTG offspring when cuttings were grown at 14°C for five weeks. For most characters, the Y x P-CDPTG progeny had greater CV's than the Y x P-Control offspring.

Table 11. Mean plant height, number of branches, number of leaves, total leaf dry weight, and total green matter dry weight of the progeny resulting from the low temperature during pollen tube growth experiment when rooted cuttings were grown for 5 weeks at 14°C.

Cross pollination - treatment	Number of progeny	Plant height (cm)	Number of branches per plant	Number of leaves per plant	Total leaf dry weight per plant (g)	Total green matter dry weight per plant (g)
P x Y - Control	70	33.1 (19)a[z]	6.0 (43)a	29.0 (31)a	.175 (29)a	.348 (33)a
P x Y - Cold during pollen tube growth	100	33.3 (19)a	5.7 (43)a	27.8 (34)a	.178 (38)a	.355 (42)a
Y x P - Control	112	33.9 (20)a	5.0 (48)b	25.1 (34)a	.175 (33)a	.359 (35)a
Y x P - Cold during pollen tube growth	55	35.4 (20)a	4.2 (52)a	23.1 (37)a	.164 (38)a	.345 (40)a

[z]Means indicated by the same letter within one pair are not significantly different according to T-test ($\alpha = .05$).

( ) Coefficient in variation.

DISCUSSION

Pollen aging in a dark, sealed vial for one week at ~25°C was the most effective treatment for producing a Y x P progeny population with an increased mean plant size and a reduced CV when compared with the control Y x P population. This suggests that the drop in P pollen viability due to aging (55% to 15%) was not random among the pollen grains and pollen survival was at least partially dependent upon its gametophytic genotype. Mulcahy et al. (1982) reported similar results with petunia. Petunia plants derived using aged pollen were significantly heavier than plants derived using fresh pollen. Puri and Lehman (1965) suggest that reserve food material in the pollen, permeabilty 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 mortality rates in storage. For example, germination and tube growth of maize (Zea mays L.) pollen after storage was influenced by loci which control carbohydrate and amino acid content and distribution in pollen (Pfahler and Linskens, 1970a).

It is also possible that the pollen aging treatment favored those alfalfa pollen grains which were more heterotic. Simon and Peloquin (1976), working with 2N=2X potato pollen formed by first division restitution (FDR) or by second division restitution (SDR), found that FDR microgametophytes had higher survival in storage than SDR gametes. Furthermore, these FDR gametes were found to posess higher breeding values than SDR gametes on the basis of tuber yield in tetraploid by diploid potato crosses (Mok and Peloquin, 1975). In both cases, the superiority of the FDR gametophytes was attributed to microgametophyte heterosis. Busbice and Wilsie (1966) suggest that heterosis is common in the gametophytes of alfalfa and other autotetraploids and vigor at the gametophytic level and the zygotic level are positively correlated. It is possible that the apparent increase in plant size of the Y x P-Aged alfalfa progeny over the Y x P-Control progeny is due to selection for heterotic P pollen grains (1N=2X).

The 29.5°C (high temperature) treatment applied either during microsporogenesis (HDM) or pollen tube growth (HDPTG) resulted in P x Y populations which were taller than the controls when grown at 30°C. Similar results were also observed at 22°C (data not presented). Genes conferring fitness to the Y microgametophytes subjected to 29.5°C temperature may be associated with plant height since this characteristic increased in both heat treatments. The

29.5°C (high) temperature treatment also appeared to influence rate of subsequent seed germination. Seed of the P x Y-HDM and P x Y-HDPTG populations germinated earlier than seed of the P x Y-22°C (control) temperature treatments. Seed of the Y x P-HDM, on the other hand, germinated later than the Y x P-control seed. Zamir et al. (1982 & 1983) suggested that sporophytic shifts resulting from a microgametophytic selection scheme are due to chromosome segments conferring different fitnesses to the haploid genome in different environments.

The pollen air drying (24 hours) treatment resulted in a Y x P-Air Dried progeny population with significantly smaller character means and larger CV's than the Y x P-Control population. Therefore, air drying was not a positive selection pressure even though a reduction in pollen viability resulted from the air drying treatment. In addition, the CV's suggest that the selection pressure was greater among the control P pollen grains than the air dried P pollen. One explaination for these results is that high pollen competition levels during pollen function (germination and tube growth) permitted a narrower subpopulation of gametophytic genotypes to achieve fertilization than would at lower pollen competition levels. In this study, pollen competition in the style was believed to be less intense when air dried pollen (low viabilty) was used as opposed to control pollen (high viabilty). In several studies, pollen competition levels were positively

correlated to vigor and/or negatively correlated to variability in the subsequent sporophytic generation (Lewis, 1954; Mulcahy and Mulcahy, 1975; Mulcahy et al., 1975; Ter-Avensian, 1978). This being the case, the Y x P-Air Dried progeny population would be expected to have a greater frequency of poor plants, shifting the progeny mean downward and increasing the variabilty since the natural selection associated with pollen competition would be lessened.

The 15°C (low temperature) treatments applied during microsporogenesis or pollen tube growth had little effect on the resulting sporophytic generation. The only mean character shift observed was a reduction in branch number for the Y x P-15°C (Cold) During Pollen Tube Growth population. As 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 Den Nijs 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 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 an increase in the number of smaller plants which the researchers suggest is the result of low competition levels due to poor pollen germination.

Microgametophytic selection at high (30°C) and low (15°C) temperatures yielded greater progeny differences when

the selection pressure was applied during pollen tube growth as opposed to during microsporogenesis. Zamir and Vallenjos (1983) observed similar results within tomato. The cold temperature effect 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 significant character shifts in the Y x P progeny. This was not entirely unexpected since freezer storage did not significantly reduce P pollen viability (38 to 23%). The selection pressure may not have been applied for a long enough period to be effective. Although Y pollen did have a substantial decrease in viability during freezer storage (81 to 25%), P x Y offspring did not occur in sufficient numbers for progeny testing. Thus, more freezing treatments would be necessary to test the effectiveness of freezing as a gametophytic selection pressure.

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 was found to differ from the control offspring, there was never a corresponding shift when the treatment was applied to the microgametophytes of the other clone. For example, only the Y x P progeny populations resulting from aged pollen and pollen subjected to 15°C (low) temperature during pollen tube growth differed significantly from the controls.

Likewise, 29.5°C (heat) applied during pollen tube growth resulted in a shift in the P x Y progeny characters but had no effect on the Y x P progeny.

The Y alfalfa clone of Russian origin is believed to be less adapted to high temperature than the P clone of Mesopotamian origin (Sinskaya, 1950). In this study, this sporophytic difference in adaptability may also be present in the gametophytic generation. Y pollen which had undergone microsporogenesis at 29.5° (high temperature), although not differing significantly in % germination in vitro from the control pollen, had many pollen grains with abnormal tube growth. Furthermore, Y pollen (developed at 22°C) which was germinated at 29.5°C had a high frequency of short pollen tubes as compared to pollen of the same sample germinated at 22°C. These results suggest that the Y micro-gametophytes are poorly adapted to high temperatures. Nevertheless, pod set of the P x Y crosses at 29.5°C was high indicating that this treatment did not effect the Y pollen's pollination ability. P pollen, on the other hand, had increased pollen tube growth at the higher temperature with good pod set of the P x Y-HDM and P x Y-HDPTG crosses. This suggests that the P pollen is more heat tolerant.

Barnes and Cleveland (1972) suggested that pollen tube length at high temperatures (25 and 30°C) may be under genetic control. It is possible that the population from which the P clone was derived may have already undergone genetic selection for heat tolerant gametophytes and the Y

clone is segregating for gametophytic heat tolerance. Furthermore, the background of the parents would suggest reciprocal results for low temperature tolerance. The only gametophytic differences noted at low temperatures was the large frequency of aborted (empty) P pollen grains resulting from the 15°C (cold) during microsporogenesis treatment. This pollen abortion may be a reflection of poor low temperature adaptation in the P clone. However, pollen tube length differences in the cold (15°C) during pollen tube growth experiment may have been overlooked. Poor germination of all pollen groups in this experiment allowed only a few pollen tubes to be observed.

The results in this research 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 treatments. In one case the reduction in pollen viabilty seemed to be influenced by gametophytic genotype while in the other it seemed random with regard to gametophytic genotype. In the temperature selection schemes where pollen viability did not appear to be significantly affected by the treatment, the genotype of the sporophyte appeared to strongly influence the outcome. This supports the statement of Maisonneuve et al. (1986) that the lack of success for pollen selection for cold

tolerance in their experiments compared to the success of the crosses reported by Zamir et al. (1982) was possibly due to the differences in the parents used. The differences in cold tolerance between the parents Zamir et al. (1982) used were large, interspecific differences. In this work, the low and high temperatures were chosen from the work of Barnes and Cleveland (1972). Therefore, further refinement of the stress temperatures might have resulted in more effective selection pressure. Finally, since alfalfa is a highly heterozygous autotetraploid, in order for the progeny population character means to be significant, they had to differ substancially between groups. In the aging treatment, a significant difference was detected with only 70 Y x P-Aged and 50 Y x P-Control progeny. However, in breeding programs where large progeny sizes are grown, the population shifts may be significant and practically useful.

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ISOZYME ANALYSIS
INTRODUCTION

The alfalfa populations resulting from the pollen air drying and the pollen aging experiments were significantly smaller and larger, respectively, than the control populations. Isozyme markers were studied to determine if genetic shifts could be observed at the biochemical level between the populations.

Prx and MDH were the isozymes investigated.

LITERATURE REVIEW

Isozymes are different enzyme forms which share identical catalytic functions. When the enzyme variation is due to allelic differences at the enzyme's structural locus these forms are termed allozymes. To evaluate the isozymes present in plant tissues, zymograms, or visible fingerprints of the proteins are developed.

Several hypothesis have been proposed for the origins of isozymes. In an extensive review of the evolution of plant isozymes, Weeden (1983) presents mechanisms which would result in enzyme variation: (1) mutation occurring at an allele (often causing a change in net charge of the enzyme), (2) endosymbiosis, (3) gene duplication with subsequent gene divergence, and (4) post-translational protein modifications. It is believed that isozyme variation is important for plant survival in changing environments (Peirce and Brewbaker, 1973).

Isozymes are frequently used as biochemical markers. Unlike morphological markers, isozymes are usually nonepistatic, nonpleiotrophic, and co-dominant in

expression. Therefore, plants heterozygous for allozymes are often readily identifiable and isozyme markers can be more accurate than morphological markers. Since isozyme analysis is a relatively quick, nondestructive (on a large scale), and simple technique to perform, its use is expanding. Isozyme markers have been important in identification of cultivars (Fedak, 1974), identification of sexual (Tanksley and Jones, 1981) and somatic hybrids (Wetter and Kao, 1976), in studies of phylogenic relationships (Crawford, 1983), as additional markers on linkage maps (Tanksley and Rick, 1980), and as markers correlated to traits of interest to plant breeders and physiologists (Tanksley and Rick, 1980).

Several methods are used to perform isozyme analysis: 1) serological methods, 2) sedimentation procedures, 3) gel filtration procedures, 4) kinetic studies, 5) pH optima observations, 6) chromatography procedures, and 7) electrophoresis methods. The latter technique will be discussed below.

Electrophoresis separates protein molecules by their net charge and in some procedures by size and shape. In these procedures a solution containing proteins is put in contact with the pores of a starch, polyacrylamide, or paper matrix. Then an electric field is applied and the protein molecules migrate through the field. Once the proteins have migrated

a sufficient distance to separate them, the current is removed. At that time, enzyme specific stains are applied to the matrix and banding patterns pinpointing the isozymes develop. This makes it possible to identify the isozymes present. However, there is evidence that some genetic variability may be undetected when zymograms are obtained from a single set of conditions (Moore and Collins, 1983).

Alfalfa Peroxidase and Malate Dehydrogenase Isozymes

Four apparently linked peroxidase loci have been identified in Medicago sativa L. subspecies caerulea and falcata, Prx-1, Prx-2, Prx-3, and Prx-4 (Quiros and Morgan, 1981). Each of these peroxidase loci has more than one allele and/or a null allele.

Peroxidase expression is tissue dependent. Strong activity is detected in root tissue and low activity is found in ovules and seeds. Prx-1 is not expressed in leaf tissue (Quiros and Morgan, 1981). Peroxidase activity also fluctuates with environment. Activity is highest during the hardening phase of alfalfa (Krasnuk et al., 1975).

Work on MDH inheritance in alfalfa was not found in the literature.

MATERIALS AND METHODS

Standard Scandalios (1969) starch gel electrophoresis was used for the characterization of the peroxidase (Prx) and malate dehydrogenase (MDH) enzymes of alfalfa. The procedure used in this study was slightly modified from that of Quiros (1981). Unlike Quiros (1981), only crude leaf tissue extracts, as opposed to extracts from root and leaf tissue, were used in the analysis. Thus, by using only leaf tissue, Prx-1 isozymes could not be detected. One to threee fresh leaves from flowering greenhouse grown alfalfa plants were ground in .3 mls of .05 M tris-HCl pH 7.5 for Prx detection or .3 mls of .02 M tris citric with 2% PEG plus 10 µM/ml mercaptoethanol pH 8.0 for MDH detection.

Over forty plants per treatment were twice tested.

RESULTS AND DISCUSSION

Malate dehydrogenase

No variation between individual plants was found for MDH. Of the few dozen alfalfa MDH zymograms examined, all consisted of three distal anodal bands plus a heavy staining region near the origin. No staining was observed on the gel slices from the cathodal region. Because of this lack of variation, a genetic interpretation of the banding pattern was not possible.

Peroxidase

Abundant variation was found among the zymograms for Prx (Figure 4). The cathode region had numerous heavy bands and the differences, if any, were difficult to interpret among the samples. Therefore, only the heterogeneity among the clearer anodal bands was analyzed.

Variation in the anodal banding patterns of alfalfa peroxidase enzymes have been described previously (Quiros and Morgan, 1981). Bands corresponding to the three alfalfa Prx-2 and two alfalfa Prx-3 allozymes (Prx2-5, Prx2-1, Prx2-2, Prx3-1, and Prx3-2), named by Quiros and Morgan, were

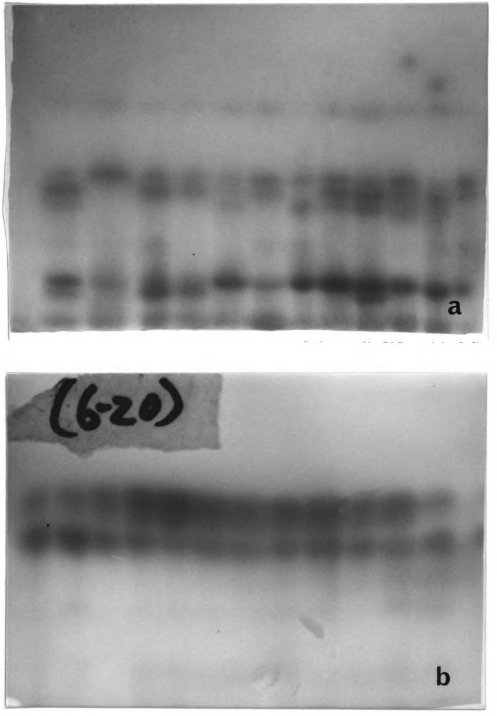

Figure 4: Peroxidase zymogram for Y x P alfalfa: a. anode region b. cathode region

found. In addition a band was usually found at the extract front and an inconsistently appearing band was found between the regions of the faster migrating Prx2 isozymes and the slower Prx3 isozymes (The inconsistent band not always being present from day to day for extracts from the same plant).

A model of inheritance of these alleles was constructed based on parental banding patterns and segregation in the progeny. Parental genotypes were believed to be:

Purple-Prx2-5,5,1,1; Prx3-1,1,N,N(null)

Yellow-Prx2-5,1,2,N; Prx3-1,1,3,N

Quiros and Morgan (1981) have reported linkage between all four alfalfa Prx loci. No linkage was detected in this experiment. Assuming no linkage, the segregation model was contrusted assuming random chromosome assortment and bivalent chromosome pairing (Table 12).

Segregation of the Prx alleles in the progeny of the pollen aging and pollen air drying experiments fit the expected ratios according to Chi Square analysis. There was no evidence of skewed distributions. Therefore, the Prx loci did not indicate a genetic shift had occurred in the progeny of the pollen aging or air drying treatments.

Table 12: Distribution of alfalfa progeny of crosses involving aged, air dried, and fresh (control) pollen for Prx-2 and Prx-3 allozymes

Genotype		Percent observed / locus				
		Theoretical ratio	Aging Expt.		Air Drying Expt.	
Locus	Alleles	distribution[w]	Fresh	Aged	Fresh	Dried
Prx2[x]	5,1,2,-[y]	39	25	40	36	42
	5,1,--[z]	44	56	45	44	44
	1,2,--	6	8	4	4	15
	5,2,--	6	8	6	4	0
	5,---	3	2	4	7	0
	1,---	3	0	0	4	0
Totals		101	99	99	99	101
Prx3[x]	1,3,--[y]	47	50	43	47	51
	1,---[z]	50	42	57	49	46
	3,---	3	8	0	4	2
Totals		100	100	100	100	99

[w]tetrasomic inheritance with random chromosome assortment and bivalent pairing of chromosomes. Parental genotypes assumed to be 1) Purple: Prx2-5,5,1,1; Prx3-1,1,Null,Null, 2) Yellow: Prx2-5,1,2,Null; Prx3-1,1,3,Null

[x]Prx2 and Prx3 allozymes as described by Quiros and Morgan (1981)

[y]genotype of yellow (maternal parent)

[z]genotype of purple (paternal parent)

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