INTERSPECIFIC POLLINATIONS OF PERENNIAL AND ANNUAL *MEDICAGO* SPECIES

by

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PART I

IN VITRO CULTURE OF PODS FROM ANNUAL AND PERENNIAL *MEDICAGO* SPECIES

ABSTRACT

Embryo abortion has been one of the main barriers to interspecific crosses between annual and perennial *Medicago* species. Since most hybrid embryos abort prior to the age at which they can be cultured, my objective was to grow young pods *in vitro*. Experiments were designed to study the nutrient requirements of the involved *Medicago* species to serve as a guide for developing a medium for hybrids. Various media were used to determine their suitability for growing pods of the following species: *M. sativa* L., *M. falcata* L., *M. disciformis* DC., *M. blancheana* Boiss., and *M. scutellata* (L.) Mill.

On media containing modified Hoagland's solution plus varied sugar concentrations, pods of tetraploid *M. sativa* produced more seeds on 4 or 6% than on 2% sucrose. Medium DM1 was superior to media containing modified Hoagland's solution plus sugar for growing 3-day-old pods of *M. sativa*.

The *Medicago* species by medium interaction was significant. Differences between annuals and perennials and diploids and tetraploids were also significant. Three-day-old pods on DM medium reached maturity in about 30 days. Over 80% of the pods from annual diploids, *M. disciformis* and *M. blancheana*, produced seeds on DM medium. In contrast to the annuals, only about 15% of the pods from perennial diploid, *M. falcata*, produced seeds on the DM medium. Although this medium was less suitable for tetraploids than diploids, 48 and 63% of the pods from annual *M. scutellata* and perennial *M. sativa*, respectively, produced mature seeds.

The addition of 1 ppm of indoleacetic acid to the DM medium (DM1) increased the number of seeds per pod in *M. sativa*, but not in the annuals. The addition of 1 ppm of gibberellic acid to the DM medium (DMG) failed to increase seed production of any species tested and significantly decreased it in annuals.

Partial pollen-stigma incompatibility and post-fertilization ovule abortion were the main barriers in interspecific crosses between perennial and annual Medicago species (Sangduen et al., 1983b). Ovule abortion also caused low self-fertility (Sayers and Murphy, 1966) or self-incompatibility of some cultivated alfalfa clones (Cooper and Brink, 1940). Sangduen et al. (1983a) determined that insufficient nutrient breakdown and transport in all nutritive tissues at the early developmental stage of the embryo contributed to abortion of ovules in wide crosses. Therefore, growing young embryos in vitro to meet their nutritional requirements may overcome those barriers and result in successful hybrid seed production.

In vitro embryo culture has been used to overcome: (1) self sterility, (2) failure of certain interspecific or intergeneric crosses in situ which might result from the lack of endosperm, and (3) seed dormancy or to accelerate seed germination. It also has been used to study physiology of reproduction (Maheshwari and Rangaswamy, 1963; Narayanaswamy and Norstog, 1964; Raghavan, 1977). Rearing the ovule or the entire ovary in vitro may be more successful than embryo culture. Rao and Rangaswamy (1972) suggested that pistil culture may prevent the precocious abscission of the ovary. Richards and Rupert (1980) stated that removing the reproductive organs could aid in avoiding post-fertilization barriers. Maheshwari and Rangaswamy (1963) reviewed successful examples of orchid, opium poppy, citrus, strawberry, bean, tobacco, tomato, gherkin, and several herbs. They indicated that degrees of success increased directly with amount of maternal tissue retained (Rédei and Rédei, 1955).

Rao and Rangaswamy (1972) suggested two conditions necessary for successful pistil culture: (1) the appropriate nutrient medium and (2) retention of the floral parts which envelope the pistil. The effect of the calyx on fruit development has been investigated in Hyoscyamus niger L. (Bajaj, 1966), Allium cepa L. (Guha and Johri, 1966), Iberis amara L.(

Maheshwari and Lal, 1961), Sisymbrium irio L. (Pareek et al.,1980), Nicotiana rustica L. (Rao and Rangaswamy, 1972) and some Trifolium species (Richards and Rupert, 1980). The contribution of the calyx was presumed to be nutritive rather than stimulatory (Maheshwari and Lal, 1961). The removal of the calyx resulted in impaired growth such as smaller fruits, decreased seed number, or seedless fruits that might be caused by the shortage of some nitrogenous compounds which were essential to fruit development (Nitsch, 1963; Guha and Johri, 1966). The addition of auxin and cytokinin to the medium induced various effects. With the addition of gibberellic acid, indoleacetic acid, and kinetin in the basal medium, Allium cepa L. developed larger fruits in vitro than those in situ (Guha and Johri, 1966). Ovary growth of Iberis amara L. and Hyoscyamus niger L. was improved by using different concentrations of IAA (Maheshwari and Lal, 1961; Bajaj, 1966). In Trifolium species, growth regulators enhanced embryo growth in culture, though the requirements varied for each species and their crosses (Richards and Rupert, 1980). Yeh and Bingham (1969) indicated that GA may be useful in enhancing the growth and seed production of some specific alfalfa stocks in vivo.

The objectives of this study were: (1) to determine the feasibility of in vitro culture of pods with attached calyx at the early development stage, and (2) to investigate the nutritional requirements as well as the effects of growth hormones GA and IAA on in vitro growth of excised pods from annual and perennial Medicago species.

MATERIALS AND METHODS

For all trials, the annual species were self pollinated by gently squeezing the flowers between fingers. The perennials were cross pollinated by using a tooth pick to trip the flower and transfer pollen grains.

Sterilization of pods was done as follows: (1) the faded corolla was removed, (2) the pod, calyx, and pedicel were placed in 10% clorox (0.525% sodium hypochlorite) solution for 10 minutes and agitated occasionally, (3) the material was rinsed in autoclaved distilled water for 1 minute, and (4) excess water was removed with a sterilized filter paper before transplanting the pods to media. Each petri dish contained about 25 ml of medium. Six or less pods were placed in each petri dish. The dishes were sealed with parafilm. All operations were performed in a laminar flow hood. Petri dishes were maintained in an incubator at approximately 25°C under fluorescent light with a 16-hour photoperiod. All the media were solidified by adding 0.8% Difco agar and were autoclaved at 121°C under 15 psi pressure for 20 minutes. The pH was adjusted to 6.0 with sodium hydroxide and hydrochloric acid.

Effect of Mineral Salts, Sugar, and Potato Extract on Growth of *Medicago* Pods *in Vitro*.

Tetraploid *M. sativa* L. (KS11) and diploid *M. falcata* L. were grown in a greenhouse. Pods were excised three through seven days after pollination. The media tested were: (a) modified Hoagland solution (Hoagland and Arnon, 1950; Johnson et al. 1957; Harper and Paulsen, 1969a,b, Table 1) plus 1% glucose; (b) (a) plus 5% potato extract; (c) modified Hoagland solution plus 1% sucrose; and (d) (c) plus 5% potato extract.

Effect of Sucrose Concentration on Growth of Tetraploid *Medicago sativa* Pods *in Vitro*.

Three-day-old excised pods from experimental synthetic KS153 were cultured on modified Hoagland's plus sucrose concentrations of 2, 4, and 6%.

About 50 pods were studied for each medium.

Effect of Medium DM1 on Growth of Tetraploid *Medicago sativa* Pods *in Vitro*.

Three-day-old excised pods from experimental synthetic KS153 were cultured on DM1 (Liang et al. 1982) (medium DM, Table 2, plus 1 ppm of indoleacetic acid). About 160 pods were studied.

Effects of Three Growth Media on Seed Production of *in Vitro* Grown Pods from Diploid and Tetraploid Annual and Perennial *Medicago* Species.

Three annuals (diploids, *M. blancheana* Boiss., *M. disciformis* DC., and tetraploid *M. scutellata* (L.) Mill.) and three perennials (diploid *M. falcata* L., tetraploid *M. sativa* L. clones SP9-16 and experimental synthetic KS79-20) were maintained in a growth chamber with an 18-hour photoperiod and a constant temperature of 24°C. Three-day-old annual and perennial pods were excised and implanted *in vitro*.

The following three media were used: (1) DM (Table 2),(2) DM1 = DM plus 1 ppm of indoleacetic acid (IAA), (3) DMG = DM plus 1 ppm of gibberellic acid (GA).

The number of pods tested for each species varied. On the average, 26 pods were grown *in vitro* for each species per replication. The number of seeds per pod was determined at maturity. The treatments were included in a completely randomized design with 13 replications except 5 replications for *M. sativa* SP9-16 x *M. sativa* KS79-20.

RESULTS

Effect of Mineral Salts, Sugar and Potato Extract on Growth of *Medicago* Pods *in Vitro*.

A few pods of tetraploid *Medicago sativa* produced viable seeds on media containing modified Hoagland's solution (Table 1) plus 1% glucose or sucrose or these plus 5% potato extract. On media containing glucose, age of pods at time of excision had little effect on seed production, but with sucrose, six-day-old pods produced more seeds than did younger ones (Table 3). Potato extract did not affect seed production.

Diploid *M. falcata* pods failed to grow on most of the media. Pods excised six days after pollination produced a few viable seeds on the medium containing 1% sucrose (Table 3).

Effect of Sucrose Concentration on Growth of Tetraploid *Medicago sativa* *in Vitro*.

The percentage of pods (excised 3 days after pollination) that produced seeds was higher in media containing 4 or 6 than on 2% sucrose (Table 4).

Approximately 39% of pods on media containing 4 or 6% sucrose produced seeds; however, 4% was superior since 23.1% of pods on that medium produced 2 seeds per pod compared to 5.6% of those on 6% sucrose.

Effect of Medium DM1 on Growth of Tetraploid *Medicago sativa* Pods *in Vitro*.

Approximately 45% of the pods produced seeds on DM1 which was higher than those on the media with modified Hoagland solution and sucrose (Table 4). Also 8.8% of pods produced 3 seeds or more on DM1 while none of those on media with modified Hoagland solution and sucrose produced more than 2 seeds.

Effects of Three Growth Media (DM, DM1, and DMG) on Seed Production of *in Vitro* Grown Pods from Diploid and Tetraploid Annual and Perennial *Medicago* Species.

The *Medicago* species responded differently on the three media (Tables 5 and 6). Also, differences between annuals and perennials and diploids and

tetraploids were significant (Table 7). For tetraploids, the difference between annual and perennial was non-significant.

Culture of pods on the DM and DM1 media was highly successful for the annual diploid Medicago species (Table 8, Fig. 1). Over 80% of M. blancheana and M. disciformis pods produced seeds on the DM medium. The average number of seeds per pod was less in culture than on the plant but three or more seeds were produced in many pods. In contrast to the annuals, only about 15% of the diploid perennial M. falcata pods produced seeds on the DM medium and the pods contained only one seed.

Although the DM medium was less suitable for the annual tetraploid M. scutellata than the annual diploids, about 48% of the pods produced 1 seed (Table 8, Fig. 1).

A high percentage (62%) of pods from the cross of two tetraploid perennials M. sativa (SP9-16 x KS79-20) produced mature seeds on DM and DM1. Although the total percentage of pods producing mature seed was similar for the two media, 16.7% of the pods on DM1 produced 3 seeds per pod compared to 1.8% on DM (Table 8, Fig. 1). The success rate (30%) for culturing pods obtained from selfing the perennial tetraploid clone SP9-16 was only half that for the cross (62%) on the DM and DM1 media.

DMG was less suitable than DM or DM1 for in vitro growth of pods from the annual diploid species (Table 8, Fig. 1). However, 66.3% of M. blancheana pods produced viable seeds and 12.2% contained 3 seeds. In contrast, only 2.1% of pods from the annual tetraploid produced seeds. More than 30% of M. scutellata pods produced a negligible number of seeds with distinctive dark brown, thickened fruit walls. Also, about 16% of the pedicels induced callus, followed by formation of the trifoliolate leaves and coil-shaped tissue. The media with or without 1 ppm of IAA, caused little callus and pods contained only one seed.

Diploid (M. falcata) and tetraploid (M. sativa) perennial species

responded similarly on DM, DM1, and DMG media.

Three-day-old pods in culture usually needed 30 days to reach maturity. The shortest period was 32-day for obtaining a germinating seed from the *in vitro* cultured three-day-old pistils of annual and perennial *Medicago* species.

DISCUSSION

Only one successful cross has been made between annual and perennial Medicago species (Sangduen et al. 1982). Fertilization occurs but embryos abort at an early age (Sangduen et al. 1983b). Removal of reproductive organs from the maternal parent may suppress post-fertilization barriers to wide hybridization.

In vitro culture of parental Medicago species may be a prerequisite to success with interspecific hybrids. Plantlets have been obtained from 8- and 12-day-old embryos of annual and perennial Medicagos, respectively, cultured on a modified Lindesmeier-Bednar growth medium (Krause et al., 1979). Unfortunately most hybrid embryos abort prior to these ages. Pod culture may be the key to success. I successfully cultured pods that were excised three days after pollination. In Allium cepa, Guha and Johri (1966) concluded that the ovary wall and placental tissue supplied unreplaceable metabolites which led to maturation of fertile seed. I included the calyx based on the successes with tomato (Nitsch, 1951) and onion (Guha and Johri, 1966). Nitsch (1963) indicated that the calyx supplies some nitrogenous compounds which are indispensible for the growth of fruits. Maheshwari and Lal (1961) concluded that the contribution of the calyx is nutritive rather than stimulatory.

Krause (1980) obtained better results with diploid annual than with perennial embryos on media. Our results with pods were similar. A high percentage of pods from the annual diploids, Medicago blancheana and M. disciformis, produced viable seeds on DM and DM1 media. In contrast, few perennial diploid M. falcata pods produced viable seeds on any media tested. The perennial species are outcrosses, so individual plant embryos are unique in genetic composition. These genotypic differences may confound embryo culture experiments. Since the annual species are self pollinated, little genetic variation occurs among the embryos and this facilitates in vitro culture.

Although the DM medium contained most of the necessary nutrients for the

diploid annuals, the requirements for Medicago species need additional investigation. In general, pod size *in vitro* was smaller than that *in vivo*. Instead of five to seven or eight coils per pod (Heyn, 1963), *M. scutellata in vitro* had four coils at most and the diameter was reduced by 25-30%. Other species I studied produced: (1) slightly smaller fruits than those *in vivo*, (2) apparently normal seedless pods, and (3) occasional small pods with one or two well developed seeds.

Component balances in nutrient media for plant growth are delicate and complex. Growth regulator requirements varied among species in *Trifolium* (Richards and Rupert, 1980). The cytokinin GA at 1 ppm did not improve pod growth or seed production of the *Medicago* species I tested. On the medium containing GA, over 30% of *M. scutellata* pods produced negligible numbers of seeds with distinctive dark brown, thicked fruit walls. Also, about 16% of the pedicels induced callus, followed by formation of trifoliolate leaves and coil-shaped tissue.

Although I identified differences between annual and perennial species and between diploids and tetraploids, this should not be interpreted as characteristic of the genus as a whole. The genus *Medicago* contains many species and thus additional studies are necessary to document differences between ploidy levels and between annuals and perennials.

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Table 1. Composition of modified Hoagland's solution.

Component	mg/l
KNO_3	505.5
$Ca(NO_3)_2 \cdot 4H_2O$	1181.0
$MgSO_4 \cdot 7H_2O$	493.0
KH_2PO_4	68.05
$FeSO_4$	2.78
Na_2EDTA	3.72
KCl	3.728
H_3BO_3	1.546
$MnSO_4 \cdot H_2O$	0.845
$ZnSO_4 \cdot 7H_2O$	0.575
$CuSO_4 \cdot 5H_2O$	0.125
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.0184

Table 2. Composition of the DM medium.

Component	mg/l
KNO_3	2500.0
NH_4NO_3	1000.0
$CaCl_2 \cdot 2H_2O$	300.0
$MgSO_4 \cdot 7H_2O$	300.0
KH_2PO_4	340.0
$FeSO_4 \cdot 7H_2O$	27.8
Na_2EDTA	37.3
$MnSO_4 \cdot H_2O$	15.5
$ZnSO_4 \cdot 7H_2O$	8.6
H_3BO_3	6.2
$CuSO_4 \cdot 5H_2O$	0.025
$CoCl_2 \cdot 6H_2O$	0.025
KI	0.830
$Na_2MoO_4 \cdot 2H_2O$	0.250
Thiamine·HCl	1.0
Nicotinic acid	5.0
Myo-inositol	100.0
Kinetin	2.0
Glycine	20.0
Lactallbumin hydrol.	500.0
Sucrose	30000.0

Table 3. Effect of mineral salt-based media on seed production of *in vitro* grown pods from two *Medicago* species.

Medium[1/]	Excised pods age	*M. sativa* (KS11)		*M. falcata* (KS94)	
		Pods	Pods produced mature seeds	Pods	Pods produced mature seeds
	days	number	%	number	%
A	4	52	3.8	—	—
	5	25	0.0	37	0.0
	6	164	1.2	146	0.0
	7	186	2.7	89	0.0
B	4	60	1.7	54	0.0
	5	77	3.9	31	0.0
	6	100	4.0	81	0.0
	7	92	2.2	50	2.0
C	3	193	1.0	141	0.0
	4	46	2.2	86	0.0
	5	28	7.1	21	0.0
	6	100	16.0	85	4.7
D	3	190	0.0	138	0.0
	4	78	3.8	89	0.0
	5	27	3.7	—	—
	6	26	11.5	28	0.0

[1/] Media were modified Hoagland's plus:

A - 1% glucose B - 1% glucose plus 5% potato extract

C - 1% sucrose D - 1% sucrose plus 5% potato extract

Table 4. Effect of media on seed production of *in vitro* grown three-day-old pods from *M. sativa* (KS153).

Medium	Seeds per pod					
	1	2	3	4	5	Total
	%					
A[1/]	15.0	5.0				20.0
B[1/]	15.4	23.1				38.5
C[1/]	33.3	5.6				38.9
DM1	18.2	18.2	5.7	2.5	0.6	45.3

[1/] Modified Hoagland's plus:

A - 2% sucrose B - 4% sucrose C - 6% sucrose

Table 5. Analysis of variance of the number of mature seeds grown *in vitro*.

Source of variation	d.f.	MS	F
Species	4	69.64	30.17**
Media	2	16.76	7.26**
S X M	8	4.44	1.92*
Days	12	1.02	0.44
S X D	48	0.97	0.42
D X M	24	0.56	0.24
Error	355	2.31	

*,** indicate significant difference at 0.05, 0.01 levels by F-test, respectively.

Table 6. Analysis of variance of the frequency of pods bearing different numbers of seeds.

Source of variation	d.f.	Mean Squares						
		P0	P1	P2	P3	P4	P5	P6
Species(S)	4	25793.1**	4383.3**	4080.2**	982.3**	99.4**	12.9**	0.4
Media (M)	2	7893.9**	3010.4**	403.3*	57.7	31.9*	8.9	0.4
S X M	8	2205.5**	1545.1**	302.8*	152.4	23.3**	8.9**	0.4
Error	180	303.3	332.5	137.4	86.2	7.4	3.4	0.4

*,** indicate significant difference at 0.05, 0.01 levels by F-test, respectively.

Table 7. Analysis of variance of the orthogonal comparisons based on the number of mature seeds grown *in vitro*.

Source of variation	d.f.	MS	F
Annual vs Perennial	1	108.50	24.43**
Diploid vs Tetraploid	1	78.20	17.60**
Annual, diploid vs Perennial, diploid	1	123.10	27.71**
Annual, tetraploid vs Perennial, tetraploid	1	0.35	0.08
M. disciformis vs *M. blancheana*	1	8.53	1.92
M. disciformis vs Perennial, diploid	1	62.52	14.07**
M. blancheana vs Perennial, diploid	1	147.30	33.16**

** indicate significant difference at 0.01 level.

Table 8. Effect of media on seed production of *in vitro* grown pods from five *Medicago* species.

Species	Medium	seeds/pod						Total
		1	2	3	4	5	6	
		%						
	DM	42.1	26.6	10.5	0.9		0.6	80.7
M. disciformis	DM1	33.2	28.3	12.2	0.6			73.3
	DMG	25.6	8.4	0.8				34.8
	DM	43.0	16.7	12.7	6.7	3.4		82.5
M. blancheana	DM1	47.4	18.8	6.2	3.7	0.5		76.6
	DMG	35.9	17.6	12.2	0.6			66.3
	DM	47.7						47.7
M. scutellata	DM1	38.9						38.9
	DMG	2.1						2.1
	DM	14.8						14.8
M. falcata KS94	DM1	8.1	1.0					9.1
	DMG	16.2						16.2
	DM	27.4	3.4					30.8
M. sativa SP9-16	DM1	28.5	2.6					31.1
	DMG	29.1	1.4	0.7				31.2
	DM	49.3	12.2	1.8				63.2
M. sativa SP9-16	DM1	30.8	14.8	16.7				62.2
x *M. sativa* KS79-20	DMG	43.2	9.0		1.8			54.0
LSD (5%)*		14.0	9.0	7.1	2.2	1.4	0.5	14.4

*Data of *M. sativa* (SP9-16 x KS79-20) were not included in analysis.

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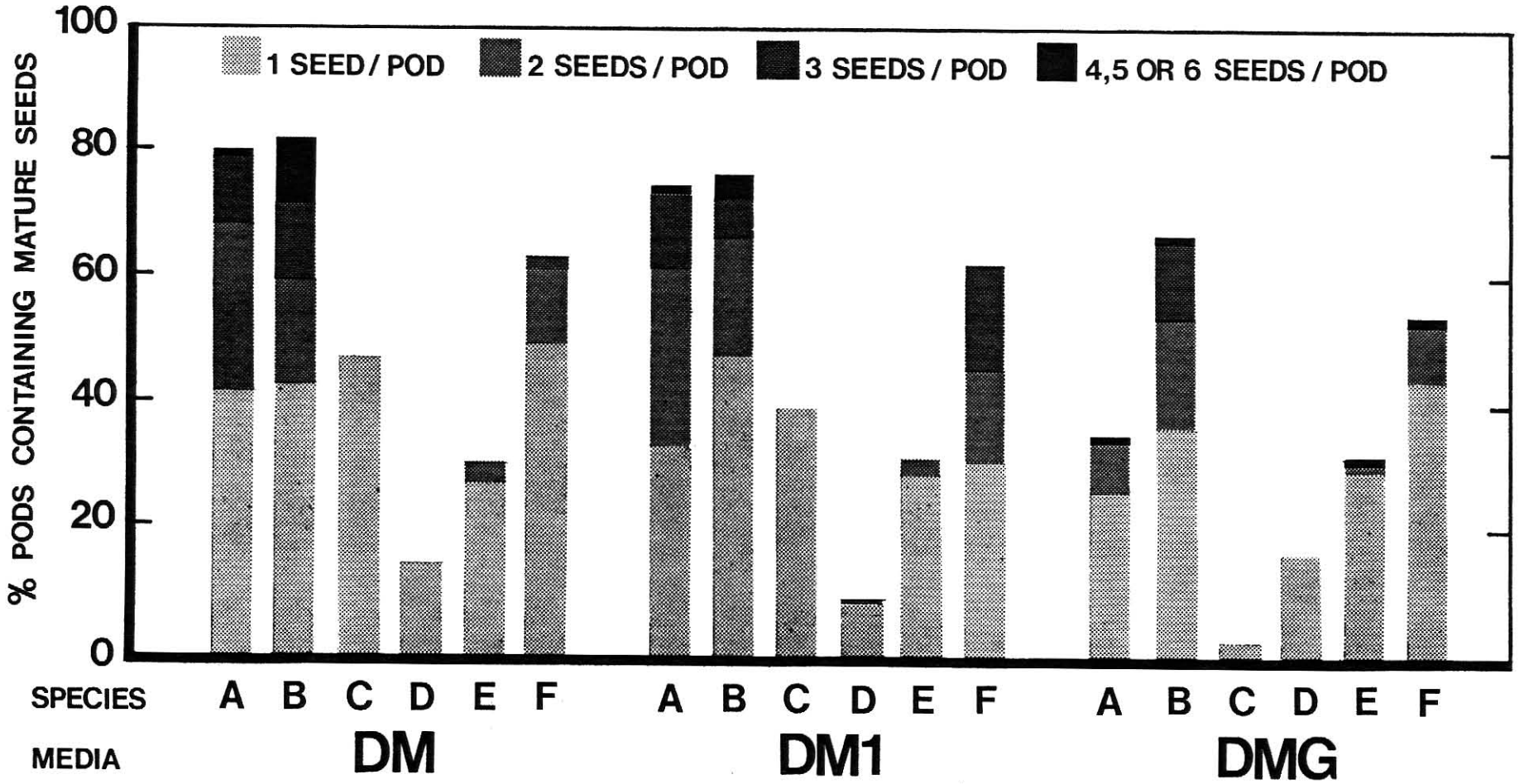

Fig. 1. Effect of media on seed production of *in vitro* grown pods from five *Medicago* species. Species A = *M. disciformis*; B = *M. blancheana*; C = *M. scutellata*; D = *M. falcata* KS94; E = *M. sativa* SP9-16; F = *M. sativa* SP9-16 x *M. sativa* KS79-20.

PART II

INTERSPECIFIC POLLINATIONS BETWEEN PERENNIAL AND ANNUAL *MEDICAGO* SPECIES

ABSTRACT

Crosses between perennial and annual Medicago species were attempted in order to transfer certain resistant characters of glandular-haired annuals into hay-type cultivars. Reciprocal pollinations were made between perennial and annual Medicago species in vivo and in vitro.

Emasculation was made for the annuals used in vivo and all pistils used in vitro pollinations. For the in vitro pollinations, pistils implanted on medium DM1 were pretreated with or without 50 ppm of gibberellic acid. Excised pods were obtained 1 to 21 days after in vivo pollination and cultured on medium DM1. Medicago species studied were perennial hexa-, tetra- and diploid sativa L., diploid falcata L., annual diploid disciformis DC., blancheana Boiss, tetraploid scutellata (L.) Mill. and rugosa Desr.

Morphological observations suggested that most plants obtained from in vivo pollinations were S_1 progeny. Seeds matured via in vitro pod culture were later identified as S_1 progeny of M. sativa, M. blancheana, and M. rugosa. Although seed was obtained from in vitro pollination, in vitro culture has high potential for yielding successful interspecific crosses. The exogenous nutrients may replace those normally supplied by the endosperm and thus prevent early embryo abortion.

Two plants obtained from the pollinations of an M. sativa clone X16-30 with pollen from M. disciformis and from M. scutellata were octoploids. Their morphological characters and polypeptide patterns were investigated. The octoploids probably were the result of a 2n female gamete uniting with a 2n male gamete. These two tetrandrogynous plants are the first documented natural octoploids in alfalfa. Different mechanisms of polyploidization might have been involved in the formation of these two octoploids.

INTRODUCTION

The glandular-haired annual alfalfa species Medicago blancheana Boiss., M. disciformis DC., M. rugosa Desr, and M. scutellata (L.) Mill. show significant resistance to potato leafhopper nymphs and adults, Empoaeca fabae (Harris)(Shade et al., 1979) and alfalfa weevil larvae, Hypera postica (Gyllenhal)(Shade et al., 1975; Johnson et al., 1980). Thus, crosses between perennial and annual Medicago species have been attempted to transfer certain insect resistant characters of the annuals into hay-type cultivars (Elgin et al., 1977; Lesins and Lesins, 1979; Krause, 1980; Sangduen et al., 1982). To date, only one mixoploid hybrid is available (Sangduen et al., 1982). My study was initiated to investigate in vitro pod culture and to extend previous experiments by using both in vivo and in vitro procedures involving more experimental clones, synthetics, and species.

Hadley and Openshaw (1980) indicated the necessity of reciprocal crosses, even though one might be more difficult than the other. Also, pollination should not be restricted by the general rule that the species with the greater chromosome number is the superior maternal parent. Reciprocal crosses, therefore, were carried out by pollinating the hand-emasculated annuals with the perennial Medicago species.

Embryo culture has been widely used to overcome interspecific and intergeneric barriers (Raghavan, 1977). Phillips et al. (1982) cultured 14- to 19-day-old embryos and obtained a hybrid from perennial Trifolium sarosiense Hazsl. x annual T. pratense L.. In Medicago sativa L., seedlings have been produced from in vitro culture of embryos excised 21 days after pollination (Fridriksson and Bolton, 1963). Krause et al. (1979) obtained plantlets of perennial M. sativa (2x and 4x) and the annuals M. disciformis and M. scutellata from 12- and 8-day-old embryos, respectively. I successfully cultured 3-day-old pods on media DM and DM1 (part I: In vitro culture of pods from

annual and perennial Medicago species). Fertilization has been observed in the perennial and annual crosses (Sangduen et al., 1983). In vitro culture of excised pods before abscission may overcome embryo abortion of these hybrid embryos.

In vitro pollination may reduce self- and cross-incompatability, induce the formation of haploid plants from ovules, and facilitate investigation of pollen physiology and the fertilization process (Rangaswamy, 1977). To carry out this technique, stigmatic, ovule, and placental pollinations have been developed. Seed formation by self-incompatible Petunia axillaris (Lam.) B.S.P. has been induced by using placental pollination (Rangaswamy and Shivanna, 1967). In the same manner, crosses between Melandrium album and M. rubrum and Silene schafta were obtained (Zenkteler, 1967). Also, in vitro pollination has been successful in selfings of the poppies Argemone mexicana, A. ochroleuca, Eschscholtzia californica, Papaver rhoeas, and P. somniferum (Kanta et al., 1962); Antirrhinum majus L. (Usha, 1965); Petunia violacea (Shivanna, 1965); Nicotiana tabacum L. (Dulieu, 1966; Zůbkova and Sladky, 1975); N. rustica L. (Rao and Rangaswamy, 1972); Agrostemma githago (Zůbkova and Sladky, 1975); and Zea mays L. (Gengenbach, 1977). This prompted my efforts to accomplish pollination for Medicago species.

Electrophoresis is a dependable tool to investigate genotypic identification, genetic variability, allelic and genotypic frequencies in a population, and to study many other plant breeding, biochemical genetic, quantitative genetic and physiological phenomena. In alfalfa, enzyme electrophoresis has been used to study the relationship of soluble proteins to the cold tolerance (Faw and Jung, 1972; Faw et al., 1976; Krasnuk et al., 1975; Krasnuk et al., 1976; Krasnuk et al., 1978); genetic variability (Vienne, 1978); and genetic polymorphisms of isozymes (Quiros and Morgan, 1981). Quiros (1980a,b) found it functions to detect heterozygotes in diploids and tetraploids. The isozyme pattern (zymogram) is formed by separating the enzymes with their different

electrostatic charges.

O'Farrell (1975) developed a powerful resolution of proteins by two-dimensional electrophoresis, which involved protein separation according to isoelectric point by isoelectric focusing (IEF) in the first dimension and according to molecular weight by sodium dodecyl sulfate (SDS) electrophoresis in the second dimension. It allows the direct detection of a large number of cellular proteins, many of which have never been previously characterized (Garrels, 1979). In plants it has been used to compare the plasma membrane proteins from the meristematic root tip and tissues of mature region in soybean (Booz and Travis, 1980) and to distinguish different cultivars of peanut by analyzing seeds and determining compositional changes that occur during seed development and germination (Basha, 1979). In addition, the appearance of different protein subunits of wheat endosperm was often correlated with a chromosome substitution (Brown and Flavell, 1981; Brown et al., 1981; Brown et al., 1979).

I report here the pollinations between Medicago annuals and perennials through in vivo and in vitro techniques. Two natural octoploids which occurred during the study were investigated morphologically and biochemically.

MATERIALS AND METHODS

Experiment (1). Two perennial male sterile Medicago sativa L. clones, 71-66-6 (2n = 6x = 48), (from E. T. Bingham, U. of Wisconsin, Madison. Wisconsin), X16-30 (2n = 4x = 32) and self-sterile HG2 (2n = 2x = 16) were hand-pollinated in a greenhouse and in a growth chamber with annual species M. disciformis DC. (2n = 2x = 16), M. blancheana Boiss. (2n = 2x = 16), M. scutellata (L.) Mill. (2n = 4x = 32) and M. rugosa Desr. (2n = 4x = 32). Conditions in the greenhouse were 22 ± 7°C and in the chamber 24 ± 2°C, 60-80% RH, and an 18-hour photoperiod.

Experiment (2). Reciprocal pollinations were carried out by dusting emasculated annuals with perennials M. sativa KS75-12 (2n = 4x = 32), BIC7 (2n = 4x = 32), CADL 15 (2n = 2x = 16), P6 (2n = 2x = 16), a yellow-flowered diploid plant with about 50% M. falcata background grown from a seed lot Wis. 72-73 (supplied by E. T. Bingham). Procedures used for emasculation were: (1) clipping the standard petal, (2) gently tripping the flower, and (3) excising the stamens with a pair of fine forceps.

Experiment (3). Pre-abscissed pods were cultured on medium DM1 (Liang et al., 1982). For the in vivo pollinated pistils, pods were excised from some crosses in experiments (1) and (2). The age of these pods ranged from 1 to 21 days. Sterilization procedures were the same as those reported in part I: In vitro culture of pods from annual and perennial Medicago species.

Experiment (4). In vitro pollination was studied as follows: Petals were removed from the excised florets. Emasculation was carried out under a dissecting microscope. Pistils with calyxes and pedicels were surface-sterilized in a 10% clorox (0.525% sodium hypochlorite) solution for 3-5 min. and rinsed twice in autoclaved distilled water. The excess water was removed with sterilized blotting papers. Sterilized pistils were implanted on medium DM1, followed by dusting the foreign pollen on the stigma. The four annuals tested

were identical to those in experiment (1). Perennials were M. sativa CADL15, HG2, KS11, KS75-12, and X16-30. Both annuals and perennials were grown in growth chambers and used as females and males.

Experiment (5). Sangduen et al. (1982) indicated that the application of gibberellic acid to pedicels induced a low incidence of self fertility in the male sterile clone X16-30. Therefore, a pretreatment was applied to the pistils by dipping them into a 50 ppm gibberellic acid for 5 min. before implanting on medium DM1. The in vitro pollination process was similar to that in experiment (4).

Two plants obtained in experiment (1), M. sativa (X16-30) x M. disciformis and M. sativa (X16-30) x M. scutellata, appeared morphologically different from the maternal parent. They were investigated as follows.

(1) Morphological characters.

Measurements were made on at least 15 samples for each character.

(2) Pollen size and stainability.

Excised mature anthers were treated with acetocarmine on slides. Pollen was squashed out of the anther by lightly tapping the cover slip. Observations were made under a light microscope at magnifications of 100X. About 450 and 100 pollen grains were studied for stainability and size, respectively.

(3) Somatic chromosome number.

(a) Root tips were pretreated in the vial with ice-cold water (TsuneWaki and Jenkins, 1960) and placed in cracked ice and water for 15 to 17 hr.

(b) Root tips were fixed in a freshly prepared mixture of 3 parts of 96% alcohol and 1 part of glacial acetic acid about 4-7 days.

(c) Root tips were placed in acetocarmine at least 1 hr. for staining.

(d) Root tips were heated in acetocarmine followed by cutting a

thin cross section on the slides.

(e) Forty-five percent acetic acid was applied to root tip cross sections and a cover slip was applied.

(f) The slides were gently heated, and the cover slips were tapped and pressed to separate the cells and spread the chromosomes.

(4) Peroxidase isozyme banding pattern.

The procedures of Othman (1983) were followed, using Hankers-Yates reagent (p-phenylenediamine-pyrocatechol). Two grams of leaves were analyzed for each plant.

(5) Two-dimensional electrophoresis of protein.

The procedure (O'Farrell, 1975) was carried out in the lab of G. R. Reeck, Dept. of Biochemistry, Kansas State University, Manhattan, Kansas.

(a) Sample preparation.

Sample extracts were obtained from the previous experiment of peroxidase isozyme banding pattern.

(b) First dimension.

Isoelectric focusing gels of 16 cm length were made in glass tubing (200 mm long x 2.5 mm inside diameter) with a gel mixture. To make 10 ml gel mixture, I used 9.2 Murea (5.5 g), 4% (1.35 ml of 30% stock) acrylamide/methylene-bis-acrylamide, and 2 ml H_2O. The 30% stock solution of acrylamide/methylene-bis-acrylamide consisted of 28.4% acrylamide and 1.6% bis-acrylamide, 2% nonidet P-40 (2 ml 10% NP-40), and 2% Ampholines (0.35 ml ampholyte, pH range 3.5 - 10.0). The gel mixture was deaerated before adding ammonium persulfate and TEMED (N, N, N'-N'-tetramethylethylenediamine). After all gels were poured, the gels were gently overlaid with water to allow polymerization for ½ - 1 hr. The layered sample buffer under the water on the top of the gels was allowed to stand for at least 1 hour. The 10 ml sample buffer was composed of 2 ml 10% NP-40,

5.7 g urea, 0.45 ml β-mercaptoethanol, 2% Ampholines (0.4 ml acidic batch), and water. Gel tubes were then placed in the electrophoretic apparatus. A mixture of 70 µl sample and 70 µl sample buffer was loaded on top of the gel after removing water and sample buffer, and then overlaid with 20 µl sample overlay buffer. The 10 ml sample overlay buffer was composed of 5.4 g urea and 1% Ampholines (0.2 ml acidic batch).

The anode lower solution was 0.01 M H_3PO_4 and the cathode upper solution, 0.02 M NaOH was deaerated. Electrophoresis was carried out initially at 300 V. The voltage was then progressively increased to 1000 and maintained for 20 hr. The gels were removed via a 10-ml syringe connected to the electrophoresis tubes by a short piece of Tygon tubing. The gels were then equilibrated for one hr. in an equilibration buffer and loaded on the second dimension gel. Equilibration buffer was composed of 10% glycerol, 5% β-mercaptoethanol, 2.3% SDS, 0.0625 M Tris-HCl (pH 6.8) and 0.05% bromphenol blue.

(c) Second dimension.

Second dimension is the discontinuous SDS gel system. Preparation of slab gel plates was illustrated by O'Farrell (1975). Twelve percent slab gels (210 x 150 x 0.8 mm) were polymerized from 29.2% acrylamide and 0.8% methylene-bis-acrylamide. The components were 51.2 ml 30% acrylamide stock, 32 ml lower gel buffer, 44.8 ml water and 64 ml TEMED, and 200 µl 10% ammonium persulfate. Lower gel buffer was made of 36.34 g Tris-base, 0.8 g sodium dodecyl sulfate (SDS), and 200 ml water. The pH was adjusted to 8.8 with hydrochloric acid. The stacking gel which was placed on the top of the running gel was composed of 30% acrylamide (7.5 ml, 0.8% bis-acrylamide), 12.5 ml upper gel buffer, 30 ml water, 50 µl TEMED, and 150 µl 10% ammonium persulfate. The upper gel buffer,

pH 6.8, consisted of 3.03 g Tris base, 0.2 g SDS and 50 ml water. A 2 mm glass rod was placed on the top of the notch, assembled from the front and back plates, and a small piece of spacer was inserted into the top left side of the stacking gel. After the gel polymerized, the first-dimensional gel was loaded on the notch nearest the unnotched plate where the glass rod was placed, and a sample of protein molecular weight markers (From Bio-Rad) was placed in the well where the spacer was inserted. They are then sealed with the melted buffer agarose solution (equilibration buffer with 1% agarose).

Electrophoresis was carried out at room temperature at 7 milliAmps per gel for 14-16 hr. Gels were stained with a solution (0.25% Coomassie blue, 10% acetic acid, and 50% methanol) for 4 hr. and destained in a mixture of 10% ethanol and 5% acetic acid until the background was clear.

(d) Measurement of pH gradient.

The isoelectric focusing gel was cut into 1 cm sections and placed individually in a vial with 2 ml deaerated water. The vials were capped and shaken for 10 minutes before the pH was measured.

RESULTS

A. *In vivo* pollinations.

Morphological observations suggested that most plants obtained by pollinating perennial *M. sativa* with annual *Medicago* species *in vivo* were S_1 progeny, even when the maternal and paternal plants were at the same ploidy level. About 1 and 0.5% of the pollinations produced seeds from the male-sterile tetraploid *M. sativa* (X16-30) and the hexaploid (71-66-6), respectively (Table 1). Two plants among the progeny of clone X16-30 were morphologically different from the others and the parent.

Plants obtained from the reciprocal pollinations also appeared to be S_1 plants. Seed sets were 33, 14, 3, and 1%, respectively, for *M. blancheana*, *M. rugosa*, *M. disciformis*, and *M. scutellata* (Table 2).

After cross pollination, the pods cultured *in vitro* produced 2 seeds from 2- and 3-day-old pods excised from *M. sativa* clone X16-30. They were later identified as S_1 progeny of clone X16-30. Three excised pods of *M. blancheana* and *M. rugosa* matured *in vitro* and plants produced from the seeds were identified as S_1 progeny (Table 3).

B. *In vitro* pollinations.

Trials of *in vitro* pollination are shown in Tables 4 and 5. Pistils on medium DM1 died or else grew to a limited size without seed development, and later lost the chlorophyll, especially on the annuals, and then died. The pretreatment of 50 ppm of gibberellic acid induced thick fruit walls and increased pod size but appeared to have no effect on pollination or seed development *in vitro*.

C. Octoploid plants.

Both of the plants obtained from the pollination of the *M. sativa* clone X16-30 with pollen from *M. disciformis* (Oa) and from *M. scutellata* (Ob)

were octoploids (2n = 8x = 64) (Fig. 1). For plants Oa and Ob, 51 and 39 cells were examined respectivedly for confirmation of ploidy level. Their morphological characters are shown in Table 6 and Fig. 2.

Peroxidase isozyme banding pattern and a redrawn zymogram (Fig. 3a and 3b) of octoploid Ob were similar to those of tetraploid clone X16-30. Plant Oa had most of the bands from clone X16-30 but also produced several additional bands which were neither found in clone X16-30 nor M. disciformis. Fig. 4 and 5 show protein patterns of the parental meterials and the octoploid plants. The calibrators for isoelectric point and molecular weight, indicated that clone x16-30 and the octoploid shared a number of identical proteins. Although Ob also showed a pattern similar to that of clone X16-30, Oa gained and lost a few proteins in the MW range between 66.2 and 31.0 x 10^3 daltons. In Oa and Ob the polypeptides did not seem to have any observably quantitative increase due to the change of ploidy level.

DISCUSSION

Self fertility of male-sterile M. sativa clone X16-30 was higher when pollinated interspecifically than when selfed (Sangduen et al., 1982). A similar frequency of S_1 progeny was obtained when tetraploid clone X16-30 and hexaploid clone 71-66-6 were pollinated with annuals. Foreign pollen seemed to stimulate self fertility.

To obtain successful crosses between perennial and annual Medicago species, I recommend in vitro culture, even though the only hybrid was obtained in vivo (Sangduen et al., 1982). Pod culture has a promising potential because it permits culture of very young hybrid embryos. The exogenous nutrients may replace those normally supplied by the endosperm and thus prevent early embryo abortion. I cultured a 2-day-old excised pod and obtained plantlet of M. sativa on medium DM1 (Table 3).

In addition to the determination of an appropriate medium for pod growth, in vitro pollination may be a prerequisite to success with interspecific crosses. Pollen germination and growth and fertilization in vitro need additional study. Placental pollination used for Petunia (Rangaswamy and Shivann, 1967) and Melandrium species (Zenkteler, 1967) does not appear to be practical for Medicago species due to the relative inaccessability of the placenta. However, stigma, ovule, and ovary (excise stigma and style from pistil) pollinations should be investigated.

The annual Medicago species are self pollinated. It was observed in mature untripped M. scutellata flowers that some pollen grains were pressed into the stigmatic membrane and were contacting secretion (Kreitner and Sorensen, 1983). Therefore, emasculation must occur prior to the pointed bud stage of flower development to prevent self pollination. Using annuals as the maternal parents for crosses with perennials needs additional study to determine the possibility of using these crosses in vivo and in vitro.

As a result of morphological and biochemical observations, the two perennial octoploid plants appeared to be S_1 progeny rather than interspecific F_1 hybrids. Qualitatively, plant Ob showed nesrly identical peroxidase isozyme banding and protein patterns to those of the tetraploid M. sativa clone X16-30 which was used as a maternal parent. Therefore, it is reasonable to assume that this plant was derived exclusively from the tetraploid clone X16-30. Another octoploid, Oa, showed different banding patterns from those of clone X16-30. Nevertheless, it did not show any definite polypeptide spot transferred from the diploid annual M. disciformis. In fact, the peroxidase isozyme and protein patterns were similar to that of the tetraploid M. sativa clone X16-30 This octoploid may have been the result of several chromosome combinations: (1) 8 + 56, (2) 16+ 48, (3) 24 + 40, (4) 32 + 32 if genomes were transmitted as a unit. Clement and Stanford (1961) found pollen grains containing mono-, di-, tri- and tetraploid chromosome complements from a dihaploid alfalfa plants after the quartet stage. However, considering the parental materials, M. sativa (2n = 4x = 32) and M. disciformis (2n = 2x = 16), it is unlikely that 5x, 6x, or 7x gametes were produced by either M. sativa or M. disciformis. In the case of having an even genomic ratio, which was a combination of two tetraploid gametes (32 + 32), the possibility of producing a 2 x 2n = 32 gamete from M. disciformis could be eliminated since the octoploid plant did not possess any character from the annual M. disciformis. These factors led to the most likely interpretation that the two octoploids were the result of two unreduced gametes(2n) from self pollination of M. sativa clone X16-30 The designation with regard to this union of two unreduced 4x gemetes is a tetrandrogynous octoploid (Mendiburu and Peloquin, 1976). The 4x pollen and egg derived from 4x parents are tetrandroid and tetragynoid, respectively. It has been shown that alfalfa of di-, tri-, tetra-, penta-, hexa- and heptaploid can be obtained from natural crosses (Bingham, 1961; Bingham and Saunders, 1974). Presumably, an unreduced egg occurred in diploid (Bingham,

1961; Bingham and Gillies, 1971), triploid (Bingham and Binek, 1969), tetraploid (Bingham and Binek, 1969; Bingham and Saunders, 1974), and hexaploid (Bingham and Saunders, 1974) M. sativa, and diploid M. falcata (Armstrong, 1954; Cleveland and Stanford, 1959). Unreduced pollen was produced in diploid (Bingham and Gillies, 1971), tetraploid (Clement and Stanford, 1961), and hexaploid (Bingham and Saunders, 1974) M. sativa, and diploid M. falcata (Cleveland and Stanford, 1959; Bingham, 1968; Bingham and Gillies, 1971; McLennan et al., 1966). Harlan and deWet (1975) have reviewed the occurrances of polyploidization on a number of plant species. Sexual polyploidization apparently contributed to the fertility of crosses between uneven ploidy levels.

Vorsa and Bingham (1979) determined the formation of 2n pollen of diploid M. sativa resulted from the disorientation of spindles which were parallel to each other at metaphse II. This mechanism is genetically equivalent to first division restitution. Clement and Stanford (1961) indicated that abnormal cytokinesis also took place in embryo-sac development and contributed to the crossability of dihaploid and tetraploid. McLennan et al. (1966) suggested that digamy might occur and result in triple fusion during the time of fertilization. In diploid Solanum phureja, three types of abnormal spindle orientation at the second meiotic division (fused, tripolar and parallel), caused the production of 2n pollen (Veilleux et al., 1982). Other possible mechanisms could be premeiotic doubling, chromosomal replication during interphase I, the period between first and second division, second division restitution and postmeiotic doubling (Mendiburu and Peloquin, 1976). Somatic doubling was concluded to be relatively unimportant in the production of polyploids (Harlan and deWet, 1975). Evidence showed that these phenomena were under the influence of environment, and more importantly inherited (Harlan and deWet, 1975). McCoy (1982) reported that the frequency of 2n pollen from diploid M. sativa varied with environments and

between flowers of the same clone under a given condition. He also determined 2n pollen formation was controlled by a single recessive gene.

Julén (1944) reported that octoploid plants were produced from colchicine treatment of M. sativa (2n = 4x = 32) and from the F_1 plant of M. sativa (2n = 32) x M. falcata (2n = 32). Crosses within these octoploid plants also could produce octoploids. They showed distinctly inferior vitality to tetraploids after the second year of planting. The shape and size of the leaves varied considerably in different plants and the leaf length was not noticeably altered as a result of chromosome doubling. However, the serration of the leaf-margin was regularly different from that of the tetraploid. He also indicated a tendency toward higher content of crude protein and fiber in the leaves. Pollen size increased with the ploidy level, Armstrong (1954) obtained an average pollen size of 35 and 50 microns in diameter for the tetraploid M. sativa and the colchicine-induced octoploid, respectively. Octoploids were also found in tissue cultures of M. sativa var. 'Saranac' with explants of immature anthers and ovaries by Saunders and Bingham (1972). They (1974) indicated the seed size significantly increased with the level of polyploidy.

The two S_1 octoploids obtained from my study are the first documented natural octoploids in alfalfa. Morphologically, they appeared distinctly different from tetraploid clone X16-30 in leaf shape, stipule, peduncle length, pollen size and range of leaflet number, and number of floret per raceme. The octoploid seemed to be inferior in vigor. The life span of Oa and Ob leaves was shorter than that of clone X16-30. The differences in similarities of the two octoploids to clone X16-30 in polypeptide constitution and fertility indicated different mechanisms might have been involved in the formation of the octoploid plants. It also suggested that increased heterozygosity might be induced by polyploidization. Crosses between natural and tissue-culture-induced octoploids (from E. T. Bingham) and tetraploids are in progress

to determine the practical advantages.

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Table 1. Number of pods and seeds produced from interspecific *Medicago* pollinations in greenhouse and in growth chamber.

Interspecific pollinations	Ploidy	Greenhouse			Growth chamber		
		Florets	Pods	Seeds	Florets	Pods	Seeds
		number					
sativa (71-66-6) X *disciformis*	6x X 2x	1533	8	6	279	1	0
sativa (71-66-6) X *blancheana*	6x X 2x	84	0	0	203	3	2
sativa (71-66-6) X *rugosa*	6x X 4x	67	0	0	18	0	0
sativa (71-66-6) X *scutellata*	6x X 4x	374	3	2	739	1	0
sativa (X16-30) X *disciformis*	4x X 2x	3336	27	32	1123	20	16*
sativa (X16-30) X *blancheana*	4x X 2x	218	3	3	961	11	10
sativa (X16-30) X *rugosa*	4x X 4x	522	5	6	133	1	1
sativa (X16-30) X *scutellata*	4x X 4x	1407	9	9	2625	27	26*
sativa (HG2) X *disciformis*	2x X 2x	424	0	0	436	0	0
sativa (HG2) X *blancheana*	2x X 2x	13	0	0	—	—	—
sativa (HG2) X *rugosa*	2x X 4x	24	0	0	—	—	—
sativa (HG2) X *scutellata*	2x X 4x	39	0	0	462	0	0

* indicate one seed grew into a plant with 64 somatic chromosomes.

Table 2. Number of pods and seeds produced from interspecific *Medicago* pollinations using emasculated annuals as female and perennial *M. sativa* as male parents.

Interspecific pollinations	Ploidy	Florets	Pods	Seeds
			number	
disciformis X *sativa* (CADL15)	2x X 2x	95	5	27
disciformis X *sativa* (KS75-12)	2x X 4x	43	1	7
disciformis X *sativa* (BIC7)	2x X 4x	3	0	0
disciformis X P6	2x X 2x	62	0	0
blancheana X *sativa* (CADL15)	2x X 2x	32	10	41
blancheana X *sativa* (KS75-12)	2x X 4x	17	8	36
blancheana X *sativa* (BIC7)	2x X 4x	13	5	18
blancheana X P6	2x X 2x	101	31	116
rugosa X *sativa* (KS75-12)	4x X 4x	60	7	10
rugosa X *sativa* (BIC7)	4x X 4x	16	4	6
scutellata X *sativa* (KS75-12)	4x X 4x	47	1	4
scutellata X *sativa* (BIC7)	4x X 4x	22	0	0

Table 3. Number and age of *Medicago* pods cultured *in vitro* on DM1 medium.

Interspecific pollinations	Ploidy	Excised pods age (Days)												
		1	2	3	4	5	6	7	8	9	12	17	20	21
		number												
sativa (71-66-6) X *disciformis*	6x X 2x	5	14	16		2								
sativa (71-66-6) X *blancheana*	6x X 2x			19	12									
sativa (71-66-6) X *scutellata*	6x X 4x		25	37										
sativa (X16-30) X *disciformis*	4x X 2x		17[a]	64										
sativa (X16-30) X *blancheana*	4x X 2x			53[a]	5	9								
sativa (X16-30) X *rugosa*	4x X 4x			6										
sativa (X16-30) X *scutellata*	4x X 4x		27	60	37		4	4		7	6	10		
sativa (HG2) X *disciformis*	2x X 2x		14											
sativa (HG2) X *scutellata*	2x X 4x	7	53											
blancheana X *sativa* (CADL15)	2x X 2x			1[b]		1	2		3					
blancheana X *sativa* (BIC7)	2x X 4x			3	2									1[c]
rugosa X *sativa* (BIC7)	4x X 4x												1[d]	1

a, one mature S_1 seed of *M. sativa* (X16-30) b, one mature S_1 seed of *M. blancheana*

c, three mature S_1 seeds of *M. blancheana* d, two mature S_1 seeds of *M. rugosa*

Table 4. Number of Medicago interspecific pollinations in vitro on DM1 medium.

Interspecific pollinations	Ploidy	Pollinations	Reciprocal pollinations
		number	
sativa (X16-30) X rugosa	4x X 4x	9	4
sativa (X16-30) X scutellata	4x X 4x	15	12
sativa (KS75-12) X scutellata	4x X 4x	9	5
sativa (KS11) X disciformis	4x X 2x	52	57
sativa (KS11) X blancheana	4x X 2x	26	19
sativa (KS11) X rugosa	4x X 4x	9	9
sativa (KS11) X scutellata	4x X 4x	15	19
sativa (HG2) X disciformis	2x X 2x	42	39
sativa (HG2) X blancheana	2x X 2x	41	18

Table 5. Number of Medicago interspecific pollinations in vitro on DM1 medium using GA-treated pistils.

Interspecific pollinations	Ploidy	Pollinations	Reciprocal pollinations
		number	
sativa (X16-30) X blancheana	4x X 2x	3	—
sativa (X16-30) X scutellata	4x X 4x	9	3
sativa (CADL15) X disciformis	2x X 2x	12	26
sativa (CADL15) X blancheana	2x X 2x	17	16
sativa (CADL15) X scutellata	2x X 4x	14	12
sativa (X16-30) X disciformis	4x X 2x	—	3

Table 6. Morphological characters of two octoploids, Oa and Ob, and tetraploid M. sativa (X16-30).

Character	M. sativa	Oa*	Ob
Leaf			
Shape	obovate, cuneate, serrate at apex	broad-obovate, serrate at apex	broad-obovate, retuse with serrate at apex
Length/Width	2.9	1.7	2.2
Stipule	entire	entire, denate with 2-6 teeth	entire, denate with 2-4 teeth
Leaflets number	3	3 to 6	3 to 6
Type of hairs	simple	simple	simple
Flower			
Peduncle length	2.4 cm	—	1.8 cm
Florets			
Color	purple	—	purple
Keel color	dark purple	—	dark purple
Standard petal length	1.0 cm	—	1.0 cm
No./raceme	3 to 19	—	2 to 11
Pollen stainability	43%	—	37%
Pollen size	35 microns	—	46 microns
Life span	perennial	perennial	perennial

* Flower buds never reach maturity.

Fig. 1. Somatic chromosomes of two octoploids, *Oa* (A) and *Ob* (B) from *M. sativa* clone X16-30.

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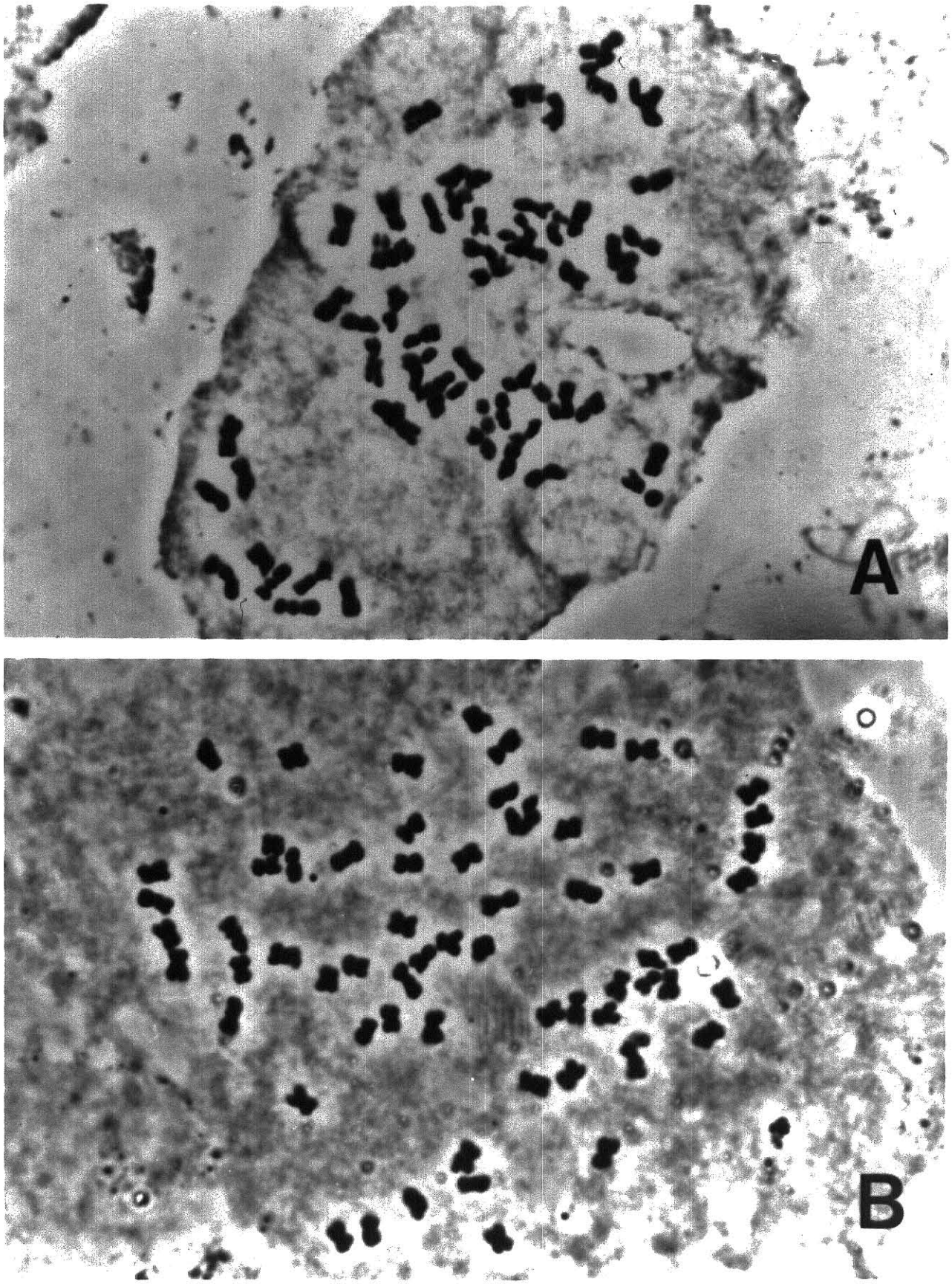

A
B

Fig. 2. Leaves of octoploid a (Oa), left, tetraploid *Medicago sativa* clone X16-30, and octoploid b (Ob).

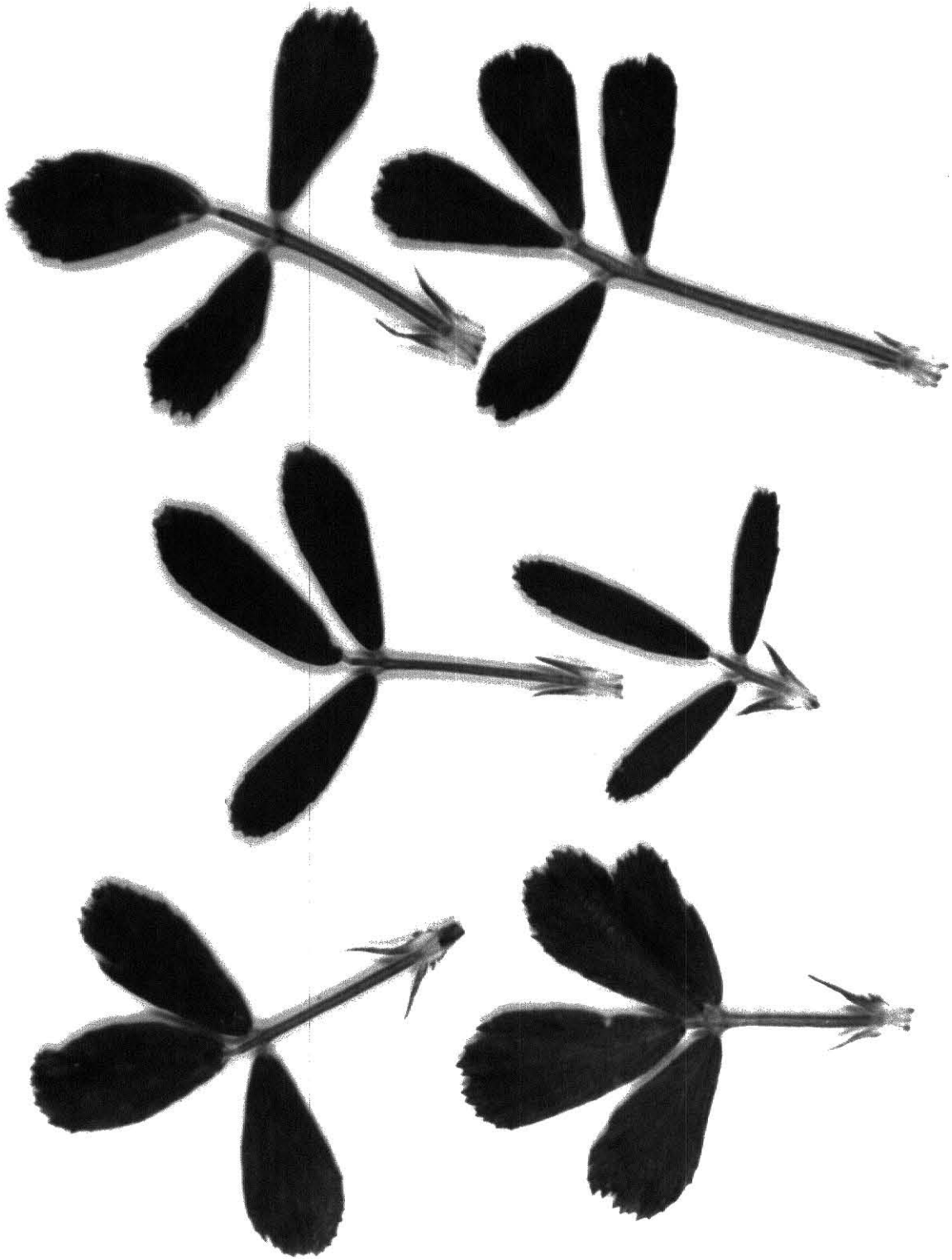
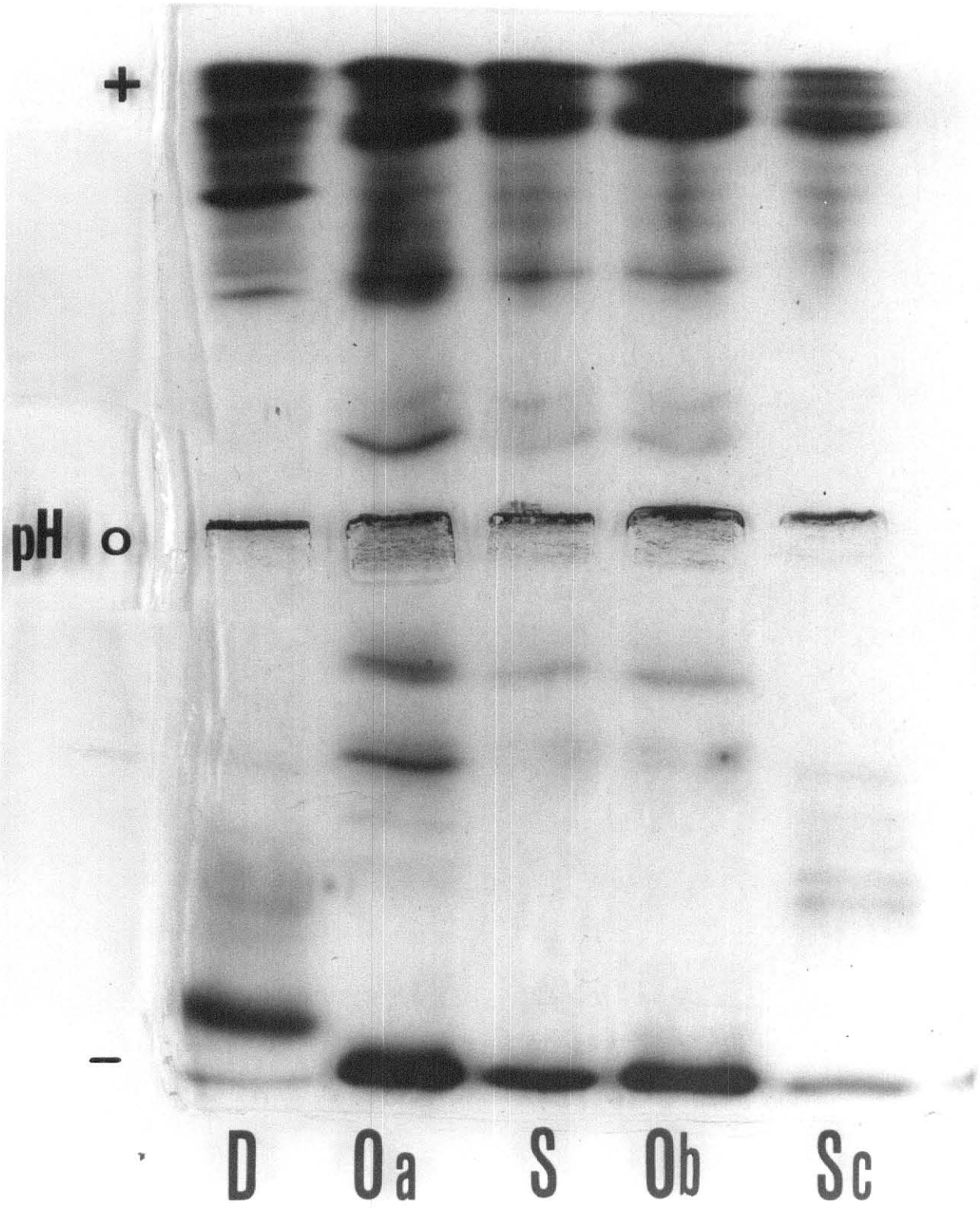


Fig. 3a. Isoelectric focusing electrophoresis showing peroxidase isozymes of the leaves from three *Medicago* species and two octoploids. D = *M. disciformis*; S = *M. sativa* clone X16-30; Sc = *M. scutellata*; *Oa* and *Ob* = octoploids.


+
pH o
−
D
Oa
S
Ob
Sc

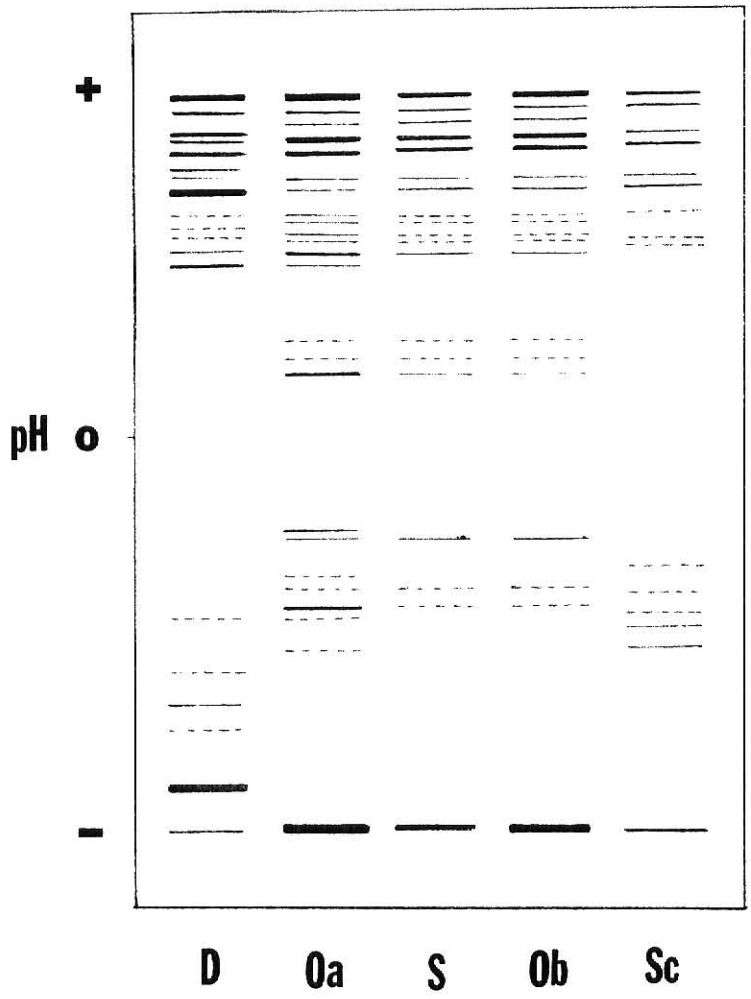
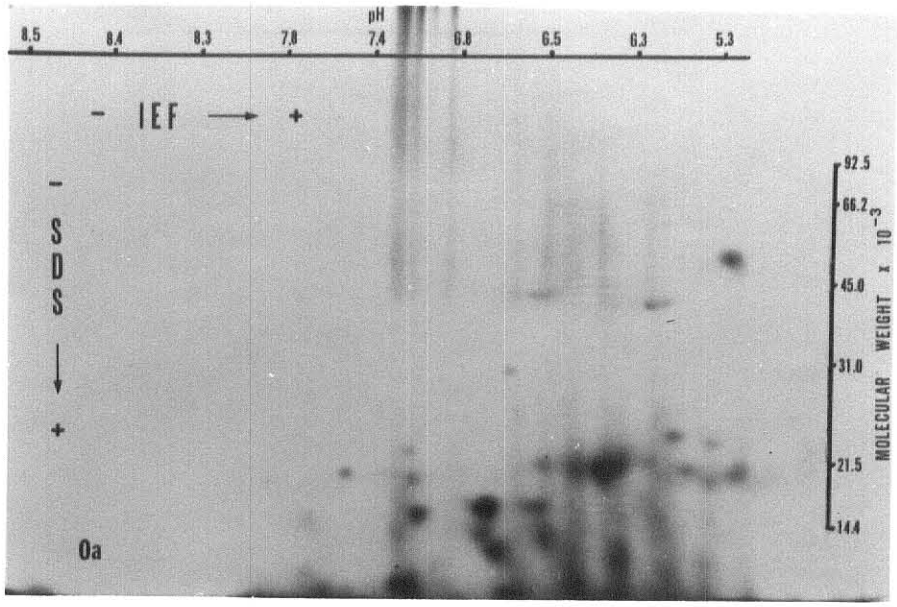
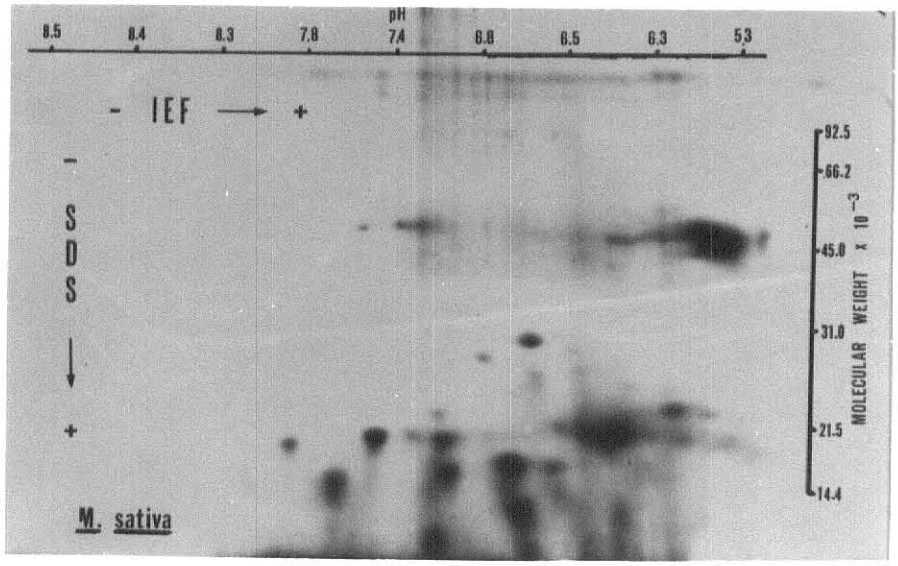


Fig. 3b. Redrawn zymogram of the gel from Fig. 3a.

Fig. 4. Two-dimensional electrophoresis protein patterns of octoploid a (Oa), M. sativa clone X16-30, and M. disciformis.


pH
8.5
8.4
8.3
7.8
7.4
6.8
6.5
6.3
5.3
- IEF +
- SDS +
92.5
66.2
45.0
31.0
21.5
14.4
MOLECULAR WEIGHT x 10⁻³
Oa


pH
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8.4
8.3
7.8
7.4
6.8
6.5
6.3
5.3
- IEF +
- SDS +
92.5
66.2
45.0
31.0
21.5
14.4
MOLECULAR WEIGHT x 10⁻³
M. sativa

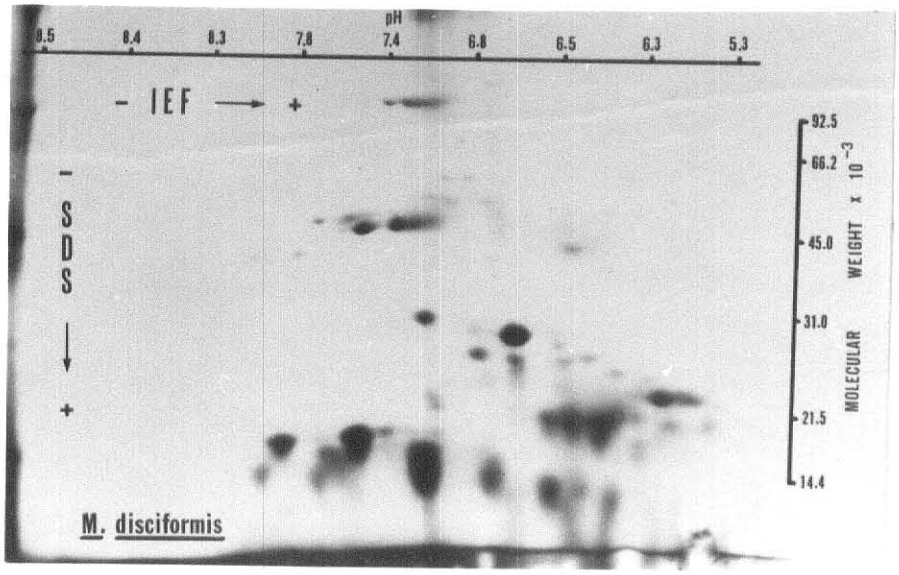
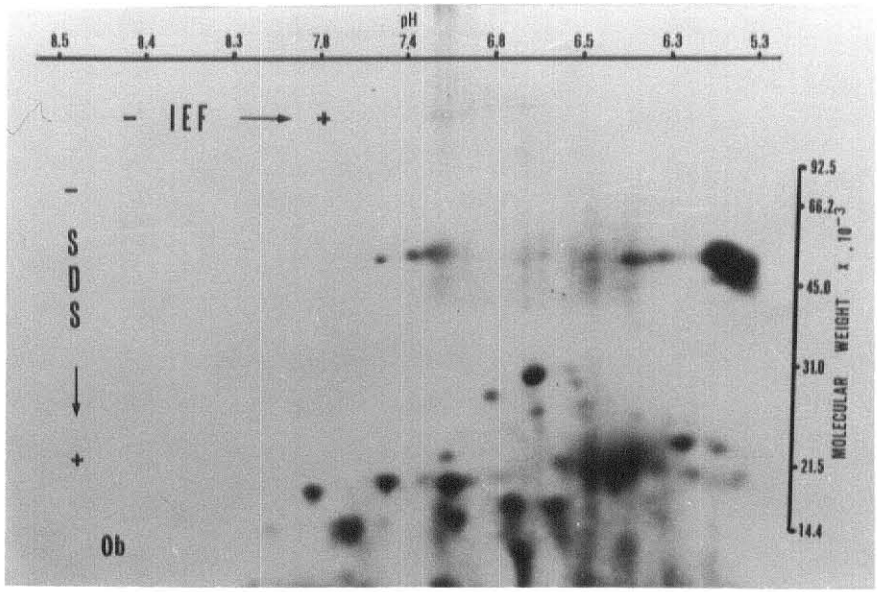
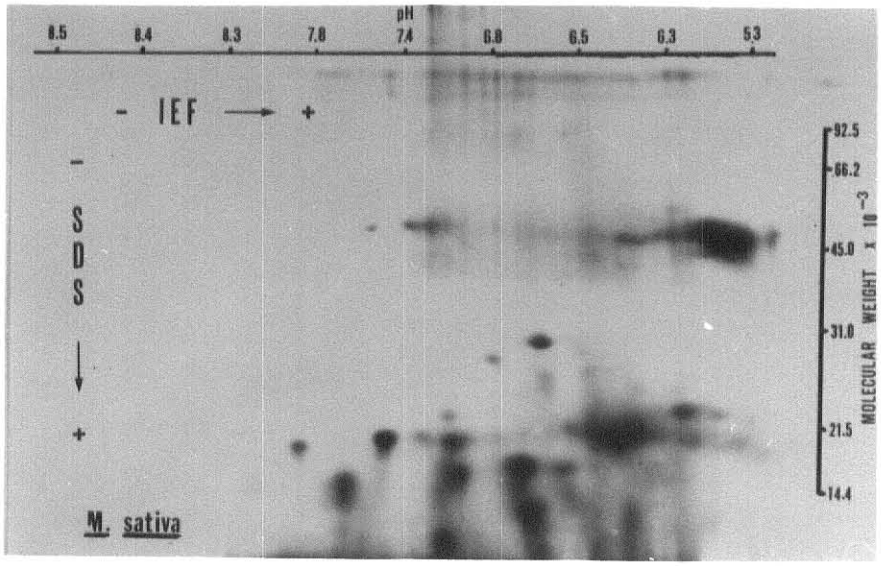
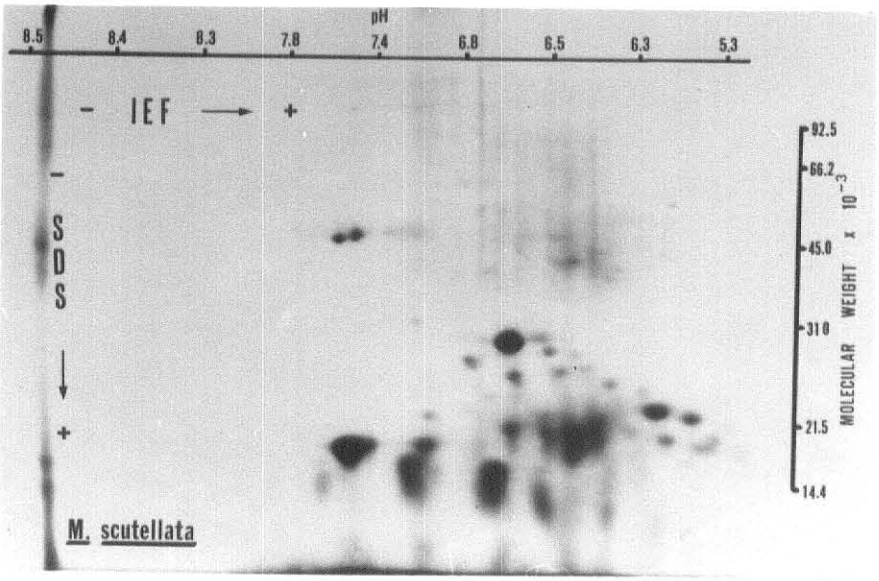

pH
8.5
8.4
8.3
7.8
7.4
6.8
6.5
6.3
5.3
- IEF +
- SDS +
92.5
66.2
45.0
31.0
21.5
14.4
MOLECULAR WEIGHT x 10⁻³
M. disciformis

Fig. 5. Two-dimensional electrophoresis protein patterns of octoploid b (Ob), M. sativa clone X16-30, and M. scutellata.


pH
8.5 8.4 8.3 7.8 7.4 6.8 6.5 6.3 5.3
- IEF +
- SDS +
92.5
66.2
45.0
31.0
21.5
14.4
MOLECULAR WEIGHT x 10⁻³
Ob


pH
8.5 8.4 8.3 7.8 7.4 6.8 6.5 6.3 5.3
- IEF +
- SDS +
92.5
66.2
45.0
31.0
21.5
14.4
MOLECULAR WEIGHT x 10⁻³
M. sativa


pH
8.5 8.4 8.3 7.8 7.4 6.8 6.5 6.3 5.3
- IEF +
- SDS +
92.5
66.2
45.0
31.0
21.5
14.4
MOLECULAR WEIGHT x 10⁻³
M. scutellata

PART III

EFFECTS OF SUCROSE CONCENTRATION ON POLLEN GERMINATION AND TUBE GROWTH OF PERENNIAL AND ANNUAL *MEDICAGO* SPECIES

ABSTRACT

Low pollen germination percentages and pollen tube growth abnormalities in interspecific pollinations of perennial x annual Medicago species prompted the investigation of pollen growth requirements. Sucrose has been used largely for in vitro pollen grain viability studies as an externally supplied nutrient and an essential osmotic regulator.

Five sugar-agar media were tested for the perennials Medicago sativa L. diploid clone CADL15 and tetraploid clone KS75-12 and for the annuals diploid M. disciformis DC. and tetraploid M. scutellata (L.) Mill. Germination percent increased directly with sucrose concentration to a maximum of 40 grams and then declined for each entry. M. sativa, clones CADL15 and KS75-12, had the highest percent germination followed by M. scutellata and M. disciformis. The percentage of burst pollen tubes was low for all genotypes. Percentage of burst pollen varied inversely with sucrose concentration. The annual diploid M. disciformis showed significantly lower germination and higher percentage of burst pollen at 10-30 grams sucrose levels than did the other species.

Percentage of pollen germination was independent of pollen tube growth. Best pollen tube growth occurred at 10-20 grams sucrose levels for M. sativa and M. scutellata. In contrast, annual diploid M. disciformis was least at 10-20 and increased to a maximum length at 40 grams sucrose level.

The presence of a genotype x sucrose concentration interaction suggested that sucrose concentration must be considered in in vitro fertilization because pollen tube length is an important criterion in cross-campatibility and fertility.

INTRODUCTION

Glandular hairs are important in plant defense, especially against phytophagus insects. Exudates from glandular hairs were implicated in resistance of some annual Medicago species to alfalfa weevil larvae [Hypera postica (Gyllenhal)](Shade et al., 1975; Johnson et al., 1980) and potato leafhopper nymphs [Empoasca fabae (Harris)](Shade et al., 1979). Inability to cross annual and perennial species has precluded the use of annuals in the development of a glandular-haired hay-type alfalfa (Elgin et al., 1977; Krause, 1980; Sangduen et al., 1982).

Swaminthan (1955) successfully overcame cross-incompatibility by placing foreign pollen on decapitated styles of Solanum species in vitro. Viable hybrids of Phaseolus coccineus x P. vulgaris were obtained by applying White's nutrient solution on the stigmatic surface prior to pollination (Ibrahim and Coyne, 1975).

Pollen germination percentages were very low and many pollen tube growth abnormalities occurred following perennial x annual interspecific crosses among Medicago species. Few pollen tubes of M. disciformis or M. scutellata grew down the styles of M. sativa L. (Sangduen et al., 1983). Sayers and Murphy (1966) indicated that the number of pollen tubes gaining entry to the ovary was controlled by the receptivity of the stigma. Sucrose has been used largely for pollen grain viability studies as an externally supplied nutrient and an essential osmotic regulator in vitro (Johri and Vasil, 1961). The low viability and growth of annual Medicago pollen on perennial stigmas may be related to sucrose requirements.

Barnes and Cleveland (1963a) found the optimum medium for diploid perennial alfalfa M. sativa and M. falcata pollen growth was 15 to 20 grams cane sugar and two grams bacto-agar in 100 ml water. Perennial tetraploid M. sativa pollen germination was highest and least variable at high sucrose

concentrations (24 to 36 grams per 100 ml water).

I am unaware of any studies regarding requirements for germination of the annual Medicago species. The objective of my study was to compare sucrose concentration requirements for germination and growth of annual and perennial pollen.

MATERIALS AND METHODS

Two perennial and two annual Medicago species were studied. Cultivated alfalfa (Medicago sativa L.) tetraploid clone KS75-12, diploid clone CADL15, tetraploid annual M. scutellata (L.) Mill. and diploid annual M. disciformis DC. were grown in a growth chamber at 23°C with 18-hour photoperiod. For each entry, pollen was collected by tripping the florets with a spear-shaped dissecting needle. A base medium was prepared by adding two grams Difco agar per 100 ml distilled water. Five sugar-agar media were prepared by using 10, 20, 30, 40, and 50 g of microbiological culture sucrose, respectively, per 100 ml of the base medium. The pH of all media was adjusted to 7.0 with sodium hydroxide and hydrochloric acid. About 1.5 ml of autoclaved medium was pipetted in a layer on a microscope slide which was placed in a petri dish containing two pieces moist blotting paper. Pollen was lightly scattered over the agar surface by using a thin smooth glass rod with a flattened end. The prepared slides were incubated in a dark chamber at 25 ± 1°C. To minimize variation of pollen tube length among plants, the incubation period was limited to six hours, since Barnes and Cleveland (1963a) found that short pollen tubes ceased their maximum growth rate between 5 and 6 hours after germination compared to 10 to 12 hours for the long pollen tubes. After this 6-hour incubation period, the slide was treated with acetocarmine (Puri and Lehman, 1965; Lehman and Puri, 1967) and placed in refrigerator to prevent further growth. Acetocarmine was used as an indication for the existence and location of the pollen cytoplasm.

Pollen germinability and pollen tube growth were studied by light microscopy at 100X. The pollen grains were placed in the following categories: (1) germinated - length of extrusive tube equal or longer than the width, (2) burst - cytyplasm partially exuded from one or more germ pores or cytoplasm diffused through pollen wall, (3) germinated with burst tube - well developed pollen tube with ruptured growing point, (4) nongerminated - length

of extrusive tube less than width.

Pollen tube length was measured with an ocular micrometer. A minimum of 100 pollen grains was measured in random microscope fields on each slide. Four slides were evaluated for each of four replications (days).

Analysis of the data was made for species, media, and interactions for each of those four categories.

RESULTS

The effect of sucrose concentration on pollen germination was similar for the annual and perennial Medicago species at the diploid and tetraploid level. The genotype x sucrose content interaction was nonsignificant (Table 1). Germination percent increased directly with sucrose concentration to a maximum of 40 grams and then declined for each entry (Fig. 1, Table 2). M. sativa (clones CADL15 and KS75-12) had the highest percent germination followed in order by M. scutellata and M. disciformis. Germination of the diploid annual species at the 40 grams sucrose level was significantly lower than that of the other species.

The percentage of pollen tubes that burst was low for all genotypes but the genotype x sucrose concentration interaction was significant (Table 1). This was due mainly to M. disciformis reacting differently than the other species. As sucrose concentration increased from 10 to 30 grams, there was a corresponding decrease in bursting of tubes for M. sativa and M. scutellata. Bursting of M. disciformis tubes remained nearly constant at these concentrations (Fig. 2). Since less than two percent of tubes burst when 40 grams sucrose was used, the best sucrose concentration for germination was not altered by subtracting burst tubes from the total germination (Fig. 3).

Lowest sucrose concentration caused the highest percent of burst pollen for each species (Fig. 4). The diploid annual had a higher percentage of burst pollen at 10 grams sucrose concentration and its response to increased sucrose was slower than that of the other species (Fig. 4). This was the principal cause of the genotype x sucrose concentration interaction (Table 1). The percentage of burst pollen was low for all species at the 40 grams sucrose concentration.

Best pollen tube growth occurred at 10-20 g sucrose concentration for M. sativa and M. scutellata. Growth decreased rapidly as the sucrose concentration increased from 20 to 50 grams (Fig. 5). In contrast, growth of the

annual diploid M. disciformis was least at 10 to 20 grams sucrose and increased to a maximum length at 40 grams.

DISCUSSION

I recorded the frequency of burst pollen to study its relationship to sucrose concentration instead of a germination/nongermination binomial response. Bursting was inversely related to the sucrose concentration. Among tested Medicago species, the relationship of high pollen bursting and low germination percentages, especially for M. disciformis, substantiate the assumption that the medium must approximate the osmotic pressure of the pollen to obtain good germination and tube growth (Schoch-Bodmer, 1936; Dean, 1964).

For the perennials and the tetraploid annual Medicago species, maximum pollen tube growth occurred at 10 and 20 grams sucrose levels, respectively. The inverse relationship between sucrose concentration and pollen tube growth agrees with the results obtained for tetraploid perennials (Lehman and Puri, 1964). Diploid annual M. disciformis pollen tubes grew best at the 40 grams sucrose level. Growth of the diploid annual pollen tubes was slower than those of the other three entries.

The range of 30 to 40 grams sucrose per 100 ml water was appropriate for the diploid and tetraploid perennials and the tetraploid annual. Pollen germination of the perennials and tetraploid annual was respectively about 25 and 10% higher than that of the diploid annual at 40 grams sucrose level. The results with diploid and tetraploid perennials and the tetraploid annual were similar to those of Miller and Schonhorst (1968) who found that, for perennial tetraploids, the percentage of pollen germinayion was independent of pollen tube growth. The unique percentage pollen germination of diploid annual M. disciformis in vitro indicates that different nutrients are required to stimulate proper growth. This may be related to the high pollen-foreign stigma incompatability in the interspecific crosses. Accordingly, the presence of genotype x sucrose concentration interactions suggested that sucrose concentration must be considered in in vitro fertilization, because pollen tube length is an important criterion in self- and cross-compatibility and

fertility as recorded by Rice et al. (1970) and Barnes and Cleveland (1963b).

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Table 1. Analysis of variance for pollen germinability and pollen tube length.

Source of variation	d.f.	Mean squares				
		Total germinated	Burst pollen tubes	Germinated	Burst	Pollen tube length
Genotypes (G)	3	2961.4**	10.6*	2756.1**	1020.9**	248.4**
Sucrose concentrations (S)	4	1716.9**	285.0**	3146.3**	1670.2**	255.5*
G X S	12	112.6	18.0**	177.6*	180.2**	64.8**
Error	60	90.2	4.0	76.6	28.3	8.8

* significant at 5% level,** significant at 1% level.

Table 2. Effects of sucrose concentration on pollen germination and tube growth of perennial and annual Medicago species.

Pollen Reaction	M. disciformis					M. scutellata					M. sativa (CADL15)					M. sativa (KS75-12)					LSD
	Sucrose Concentration (g. sucrose / 100 ml H_2O)																				
	10	20	30	40	50	10	20	30	40	50	10	20	30	40	50	10	20	30	40	50	
	percent																				
Total germinated	21.4	20.1	28.1	48.9	36.5	41.0	48.8	52.2	60.2	42.1	42.8	56.5	65.6	71.5	58.0	35.8	46.4	62.6	71.4	54.6	13.4
Burst pollen tubes	5.9	5.1	5.7	1.3	0.4	11.9	3.8	1.6	1.1	0.7	11.8	6.4	1.4	0.9	0.9	14.8	6.8	1.6	2.1	1.3	2.8
Germinated	15.5	15.0	22.4	47.6	36.2	29.1	45.1	50.6	59.1	41.4	31.0	50.1	64.2	70.6	57.1	21.0	39.6	61.0	69.3	53.3	12.4
Burst	44.1	34.3	16.5	2.4	0.4	17.7	7.6	2.2	0.6	0.1	12.5	4.7	1.3	0.4	0.1	23.7	9.3	1.9	0.7	0.2	7.5
	microns																				
Pollen tube length	44	45	69	105	67	204	191	131	97	71	201	200	113	91	64	183	202	132	89	67	42

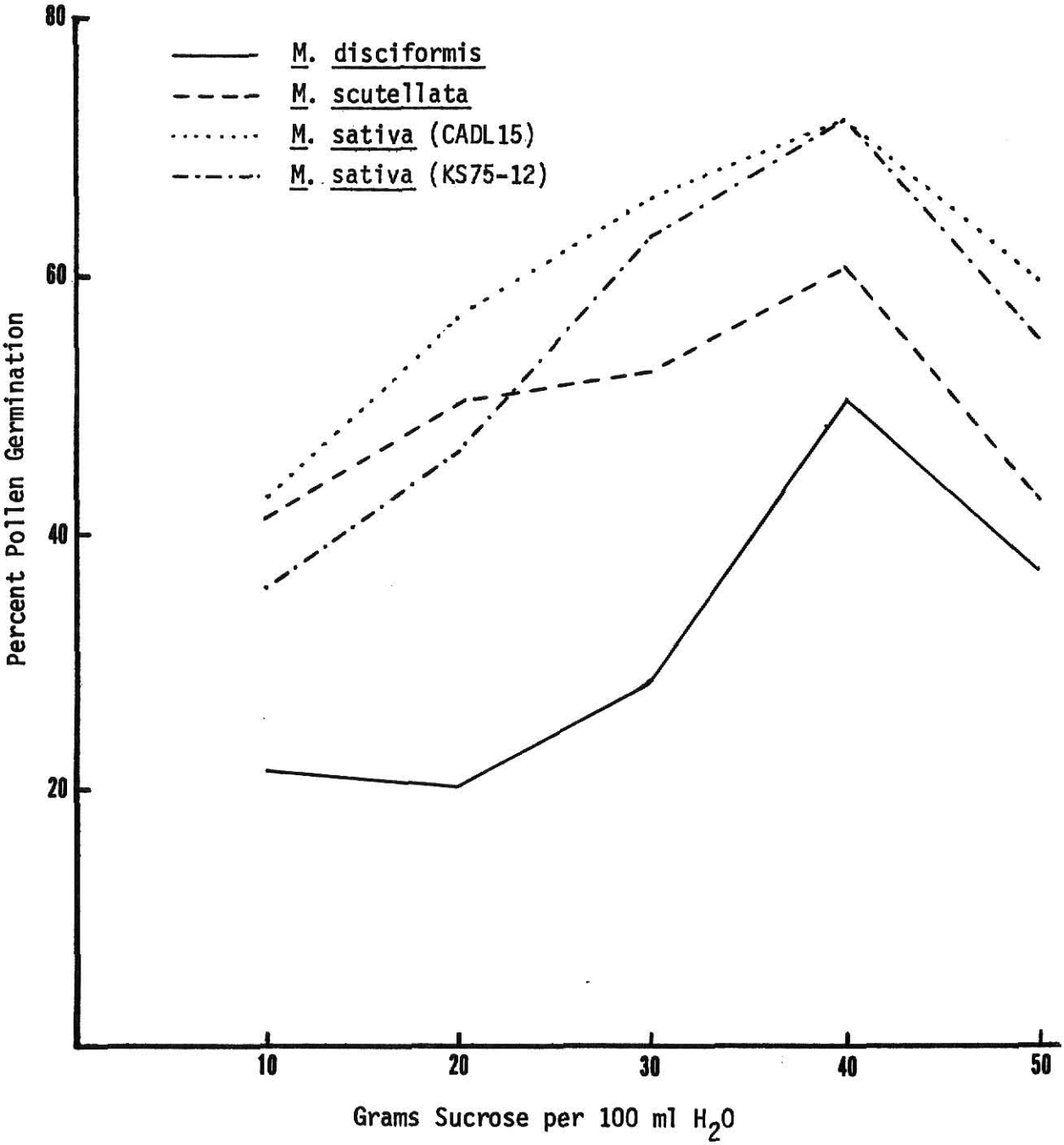

Fig. 1. Effect of five sucrose levels on pollen germination of Medicago species.

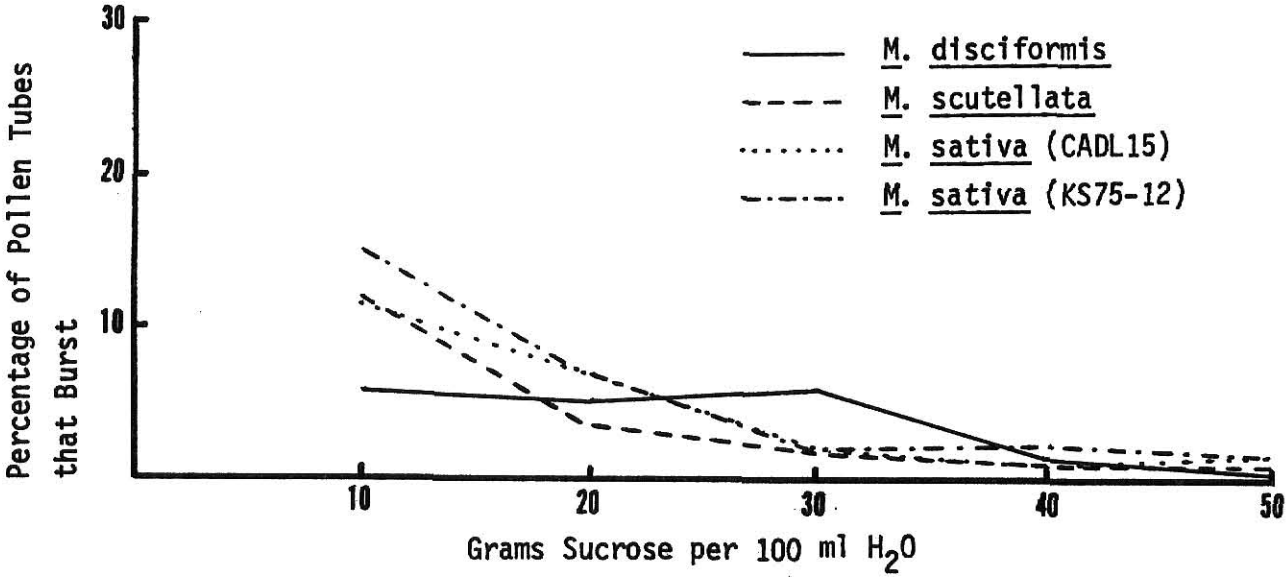

Fig. 2. Effect of five sucrose levels on bursting of pollen tubes of Medicago species.

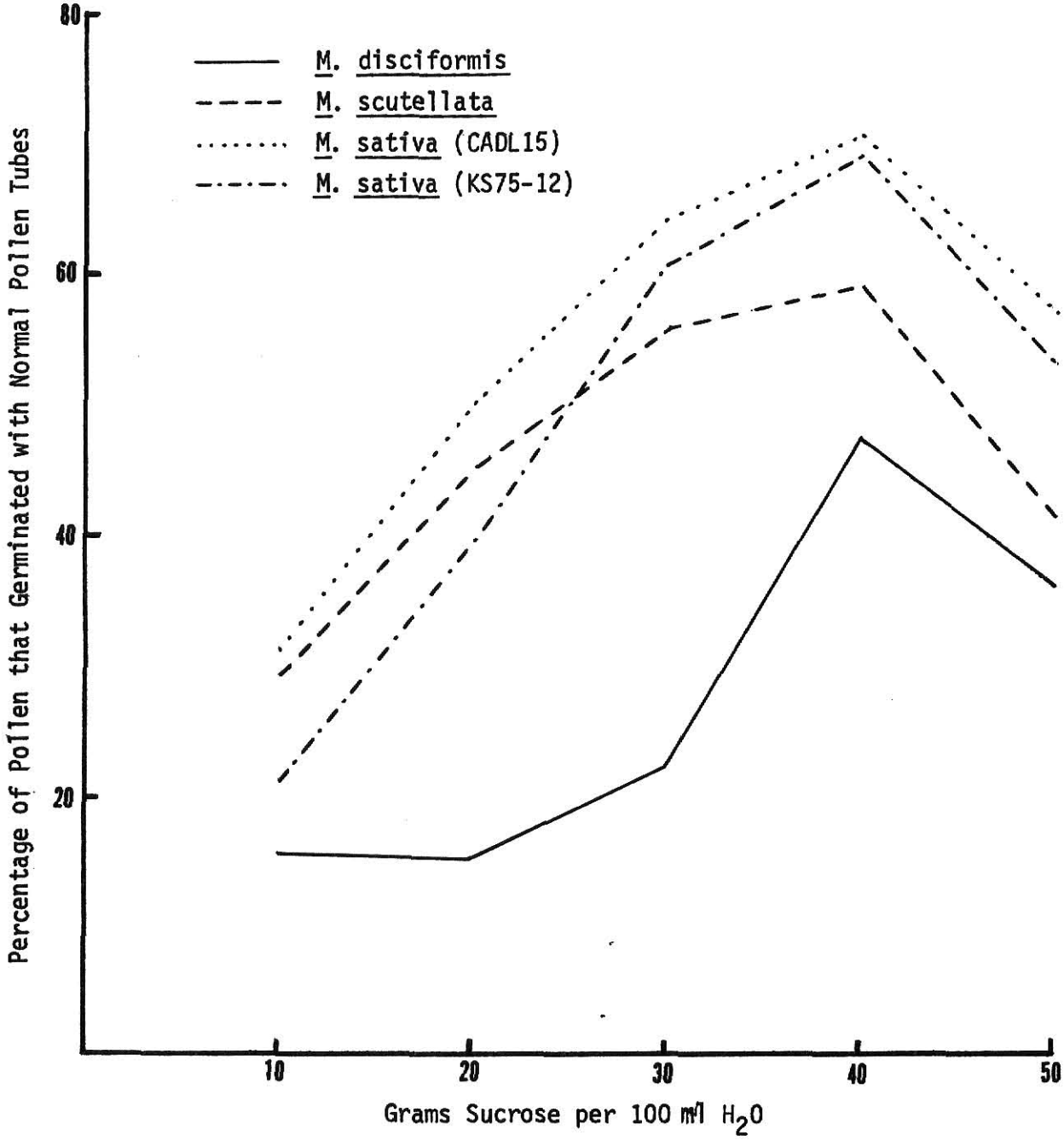

Fig. 3. Effect of five sucrose levels on development of normal tubes in Medicago species.

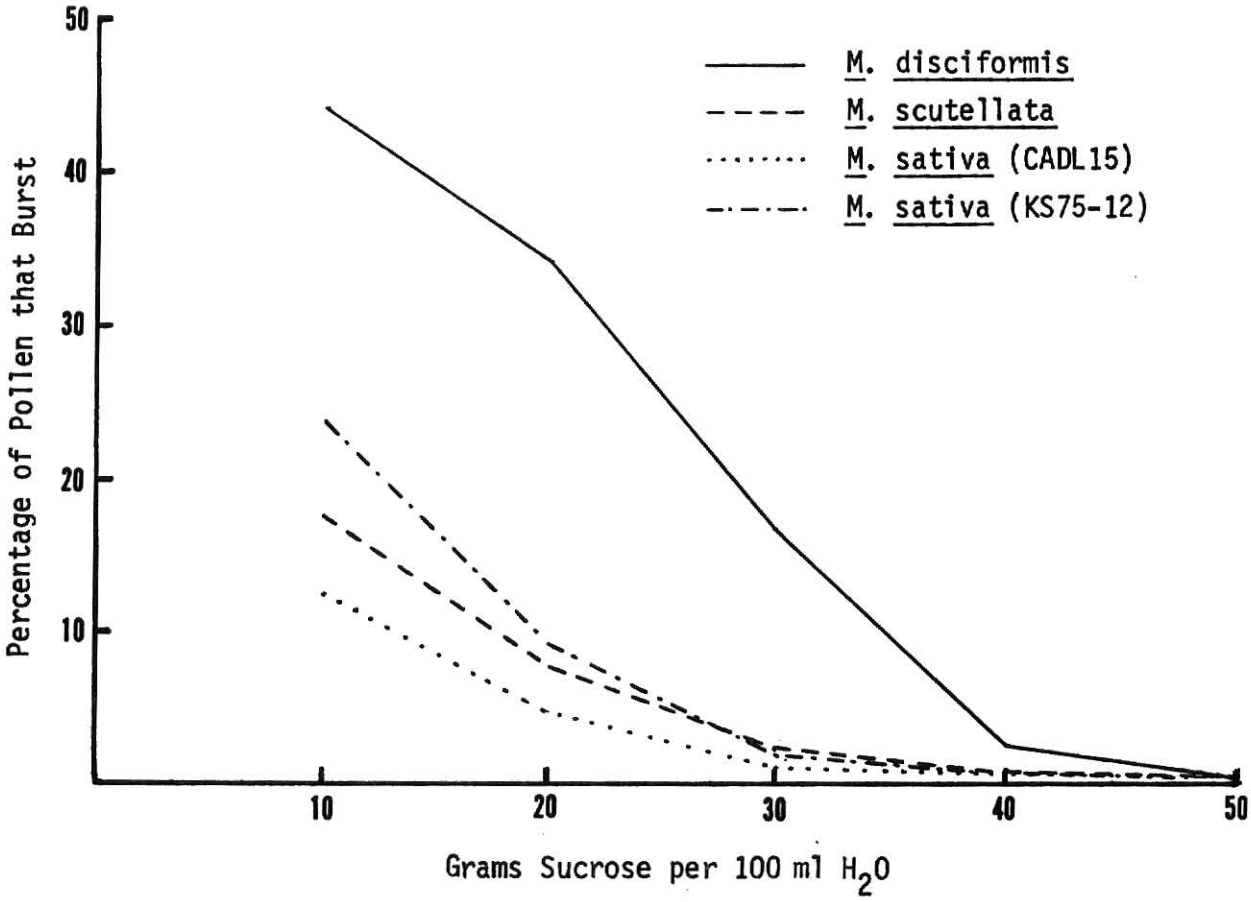

Fig. 4. Effect of five sucrose levels on bursting of pollen from Medicago species.

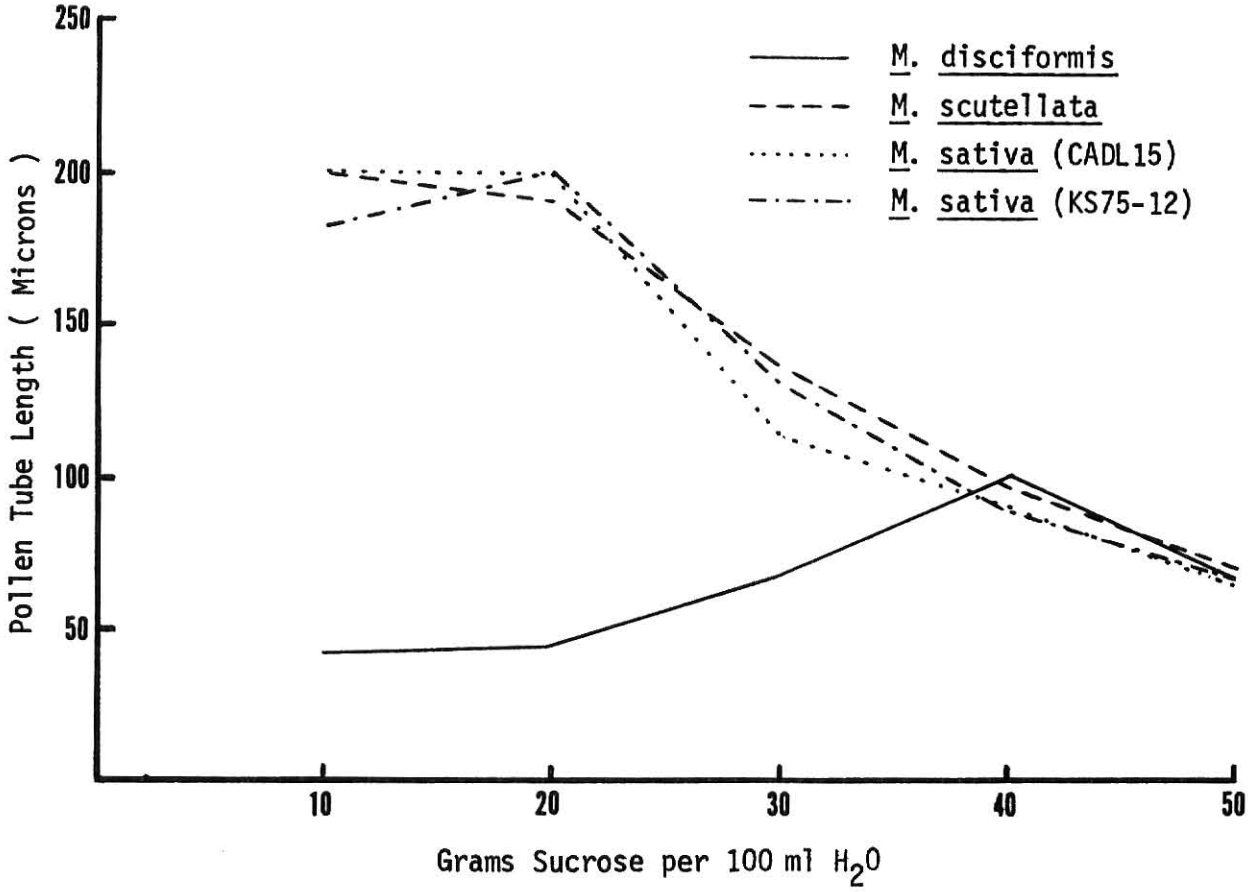

Fig. 5. Effect of five sucrose levels on pollen tube length of Medicago species.

INTERSPECIFIC POLLINATIONS OF PERENNIAL AND ANNUAL *MEDICAGO* SPECIES

by

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Certain annual glandular-haired Medicago species are resistant to alfalfa weevil [Hypera postica (Gyllenhal)] and potato leafhopper [Empoasca fabae (Harris)]. Crosses between annual and perennial Medicago species were attempted to introduce resistance into hay-type cultivars. To overcome embryo abortion and partial pollen-stigma incompatibility, in vitro pollination, pod culture and pollen growth requirements were studied.

Various media were used to determine their suitability for growing young pods. The species by medium interaction was significant. On DM medium, 83, 81, 63, 48, and 15% of pods from the species M. blancheana Boiss., M. disciformis DC., M. sativa L., M. scutellata (L.) Mill. and M. falcata L. produced seeds, respectively. DM medium plus 1 ppm indoleacetic acid increased the number of seeds/pod in M. sativa. Gibberellic acid failed to increase seed production.

In vivo and in vitro reciprocal pollinations were made between perennial and annual Medicago species: sativa, falcata, blancheana, disciformis, scutellata and rugosa Desr. In vivo pods from interspecific pollinations were excised at various ages and cultured on DM1 medium. Morphological observations suggested that most plants obtained from in vivo pollination and pod culture were S_1 progeny. No seed was obtained from in vitro pollination. Two plants obtained from in vivo pollinations were octoploids. Their morphological characters, peroxidase isozyme and protein patterns were investigated. The octoploids probably were the result of a 2n female gamete uniting with a 2n male gamete. To my knowledge, these two tetrandrogynous plants are the first documented natural octoploids in alfalfa. Different mechanisms of polyploidization might have been involved in the formation of these two octoploids.

For M. sativa, M. disciformis, and M. scutellata, percent pollen germination in vitro increased directly with sucrose concentration to a maximum of 40 grams. M. sativa had the highest percent germination. Percentage of burst pollen varied inversely with sucrose concentration. M. disciformis reacted differently than did the others with significantly lower germination

and higher percentage of burst grains at 10-30 g sucrose levels. Percent germination was independent of pollen tube growth. Best pollen tube growth occurred at 10-20 for M. sativa, and M. scutellata and 40 g sucrose for M. disciformis.