

## Self-Incompatibility in Two Alfalfa Populations

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### ABSTRACT

Self-incompatibility provides a useful mechanism for pollination control in alfalfa, *Medicago sativa* L. Five hundred randomly selected seedlings from each of two well-adapted multiple pest-resistant alfalfa populations (W10 AC3 and BMP8 AC3) were screened for self-incompatibility. Twenty-eight plants were selected following three evaluations in the greenhouse. Unemasculated hand pollinations of the self-incompatible plants, using plants with the single-gene, completely dominant red-root character as the pollen source, resulted in the production of 98.6% hybrid seed. Plants were intercrossed in the greenhouse using honey bees, *Apis mellifera* L. Growth chamber forage-yield trials of half-sib progenies indicate that certain combinations of self-incompatible plants exist that will yield significantly better than the parent populations. Twelve elite self-incompatible plants selected after the initial screening produced (i) < 0.15 seeds floret<sup>-1</sup> when self pollinated, (ii) > 30% viable pollen, and (iii) > 3 seeds floret<sup>-1</sup> when used as the male or female in crosses with other plants. Pollen-pistil interaction studies of the self-incompatible plants showed that the pollen had normal viability and either failed to germinate upon self pollination or pollen tube growth was abnormal. Pollen tubes that grew near the ovules either formed bulbous structures and terminated, or formed several branches, or continued to grow past all of the ovules resulting in the failure of fertilization. However, normal fertilization and good seed set were obtained when the self-incompatible plants were used both as male and female in crosses with other plants. The 12 elite self-incompatible plants were shown to have the capacity to produce somatic embryos in vitro, indicating that these plants could be propagated utilizing artificial seed technology. Evidence to date indicates that the use of the self-incompatibility system may provide a mechanism for successfully producing high-yielding hybrid alfalfa cultivars.

ALFALFA BREEDERS have long speculated on the value of hybrid cultivars in maximizing forage production of alfalfa fields. Childers and Barnes (1972) discussed this topic in detail and concluded that hybrid cultivar production could revolutionize the alfalfa industry. Nearly 50 years ago, Tysdal et al. (1942) discussed techniques for hybrid production. One technique they proposed involved the use of self-incompatibility, however, they concluded that the self-incompatibility mechanism in the plants they studied was not sufficiently stable, and the technique was discounted. Twenty-five years later, the concept of using cytoplasmic male sterility maintainer and fertility restorer lines in a three-way hybrid cross was introduced by Davis and Greenblatt (1967). This system was utilized to produce the first successful hybrid alfalfa cultivar (Viands et al., 1988). However, due to difficulties in obtaining sufficient seed on the male-sterile lines, attributed to the nonpreference of pollen-collecting insects for the pollen-deficient male sterile lines, hybrid cultivar production using cytoplasmic male sterility was not considered economically practical. The 10 to 15% increase in forage production from the hybrid cultivar did not sufficiently offset the significant increase in cost of seed production for the

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hybrid as compared to conventional synthetic cultivars (Viands et al., 1988).

Recently, we began to reevaluate the possibilities for hybrid cultivar production using self-incompatibility as first described by Tysdal et al. (1942). Self-incompatibility is defined in this paper as the inability to produce seed upon selfing; however, both the male and female gametes are functional when outcrossed. Self-incompatibility is of particular interest in light of the advances in technologies relating to the use of somatic embryos derived from tissue culture in the production of artificial seeds (Redenbaugh et al., 1986). Once this technology is improved, artificial seeds may be used to vegetatively propagate genetically uniform plants in seed-production fields. Self-incompatible plants could be multiplied vegetatively and planted as mixtures of the two lines in the same row. Fertile pollen would be produced on both parents but would germinate on and fertilize only the other line. Hybrid seed could be harvested from all of the plants in the field rather than from a portion of the field, as required with hybridization systems using male sterility. Even without the benefit of artificial seed technology, plant breeders could use the self-incompatible system to produce  $F_1$  hybrids in the breeder-seed generation in small plantings using conventional vegetative propagation followed by a generation of conventional seed increase, with the high probability of carrying some heterosis to the certified generation. In addition, perpetuation of self-incompatibility in generations following the  $F_1$  would allow the possible production of double-cross hybrids in sufficient and economical quantities.

We investigated the feasibility of selecting self-incompatible plants from two alfalfa populations as a mechanism for producing hybrid alfalfa with increased forage production (Elgin and Baughan, 1986). Specific objectives were to (i) select self-incompatible plants based on the results of selfing, outcrossing, and pollen viability; (ii) evaluate half-sib progenies from self-incompatible plants for their forage yield potential in a growth chamber; (iii) observe pollen tube-pistil interactions of self-incompatible plants when selfed and outcrossed to ascertain if prezygotic hybridization barriers exist; and (iv) determine the ability of self-incompatible plants to produce somatic embryos *in vitro*, for potential use as a method of asexual propagation.

## MATERIALS AND METHODS

### *Selection of Self-Incompatible Plants*

Five hundred randomly selected seedlings each from two well-adapted multiple pest-resistant alfalfa populations, W10 AC3 and BMP8 AC3 (Elgin and Ostazeski, 1984), were grown in the greenhouse at Beltsville, MD, in the spring of 1985. Greenhouse temperatures ranged from 20 to 30 °C. Tops were removed from the first growth. When the second growth reached flowering, a minimum of 30 florets on each plant were self-pollinated. Pollen quantity was estimated (1 = no pollen, 2 = small quantity of pollen, and 3 = medium to high quantity of pollen) for each plant, based on observations of quantities accumulated on a folded red plastic pot label used for self pollination. Approximately 30 d after

selfing, pods were harvested and the number of seeds produced per floret tripped was calculated.

Plants were discarded that produced more than 1 seed floret<sup>-1</sup> tripped in self pollinations or were male sterile. Self fertility of 66 putative self-incompatible plants were tested in the greenhouse twice more, using the same procedures described above. During the second evaluation period, selfing was conducted during July and August 1985, when temperatures in the greenhouse ranged from 30 to 40 °C; the third evaluation period was during January and February 1986, when temperatures ranged from 15 to 25 °C. Pollen viability was measured using cotton blue stain (Hauser and Morrison, 1964). Pollen was collected from 10 florets plant<sup>-1</sup>, stained on a microscope slide, and the percentage of viable pollen among 1000 pollen grains was determined. Only those pollen grains that stained blue and appeared perfectly spherical were considered to be viable.

Determination of female fertility of the 66 putative self-incompatible plants was made by pollinating  $\approx 30$  florets on each of the suspect plants with pollen from unrelated self-fertile plants selected from 'Arc'. These self-fertile plants were selected on the basis that they produced >75% viable pollen and >3 seed floret<sup>-1</sup> when selfed. Plants that produced no seed when outcrossed were considered to be female sterile and were thus discarded. Pollen from the self-incompatible plants was used to pollinate 30 florets of a male-sterile plant, WIS MS-2 (received from E.T. Bingham, University of Wisconsin, Madison, WI).

In addition, during the third evaluation period, pollen from a plant carrying the red-root gene (Barnes and Hanson, 1967) in the triplex or quadruplex condition was used to pollinate 20 florets on each self-incompatible plant, to determine if the presence of foreign pollen on the suspect plants stimulated the plant to accept its own pollen. A single such red-root plant was propagated and used as the pollen source. The red-root clone was selfed, to determine if 100% of the progeny would have red roots. Homozygous recessive plants have white roots. The red-root clone was also crossed to self-fertile and male-sterile plants, to check for differences in the pollen receptivity compared to the suspect self-incompatible plants. All of the progeny were planted in pasteurized, soil-filled plastic pots (10 by 10 cm) and placed in the growth chamber maintained at 24 to 27 °C with >70% relative humidity. Four to six weeks after planting, roots of the seedlings were rated for the presence of red pigmentation. Detection of the red pigmentation was enhanced by dipping the roots into a solution of 0.5 M HCl.

Twenty-eight plants exhibiting nearly complete self-incompatibility were selected for further study due to their consistent results regardless of the time of year in which the crossing occurred.

### *Evaluation of Half-Sib Progenies*

The 28 plants that exhibited self-incompatibility were placed randomly on a caged bench in the greenhouse and intercrossed using honey bees as pollinators. Half-sib seed was harvested from each plant. Progeny from the 28 plants were evaluated for their yield potential in a growth chamber study. Progeny were planted in pasteurized, soil-filled flats (52 by 30 by 7 cm) and placed in a growth chamber maintained at 24 to 27 °C with >70% relative humidity. Progeny were arranged in a randomized complete-block design with seven replicates. Fifteen seeds of each half-sib family were planted per row per replicate. Two rows each of the parent populations (W10 AC3 and BMP8 AC3) were also planted in each flat. Yield data were obtained from three harvests at 30-d intervals, starting 30 d after planting. The number of seedlings and the total weight of the forage from each was

obtained, and the average weight per plant calculated and averaged over harvests.

Twelve elite self-incompatible plants, six from each parent population, were selected from the original 28 plants for further study.

#### Observations of Pollen Tube Growth

Pollen-tube growth studies were conducted using fluorescence microscopy on the 12 elite self-incompatible plants by placing two racemes from each plant in FAA fixative (1:8:1, formalin/80% ethyl alcohol[v/v]/acetic acid) 18 h after self-pollination and 18 h after pollination by the self-fertile selections from 'Arc'. Also, racemes from a male sterile tester plant, WIS MS-2, were collected 18 h after these plants were pollinated using pollen from the putative self-incompatible plants. Following fixation for at least 24 h, the florets were softened with 1M NaOH for 8 h. Pistils were dissected from the florets and placed in a 0.1% aqueous aniline blue solution (Sangduen et al., 1983). Pistils were analyzed for (i) number of pollen grains that germinated and penetrated the stigma, (ii) number of pollen tubes in the style, (iii) average number of pollen tubes in the locule, (iv) percent ovules fertilized, and (v) average number of ovules. Observations were made using an epifluorescence microscope with a 50-W mercury lamp, with excitation at 365 nm, reflector 395 nm, and barrier filter 420 nm.

#### Tissue Culture Methods

The 12 elite self-incompatible clones were subjected to a tissue culture scheme similar to that reported by Walker and Sato (1981). Callus cultures were initiated from surface-sterilized stem sections on Schenk and Hildebrandt (1972) salts and vitamins containing 3% sucrose, 0.8% agar (SH medium), 25  $\mu$ M  $\beta$ -naphthalene acetic acid, and 10  $\mu$ M kinetin. Cultures were grown at 24 to 28 °C with a 16-h photoperiod (200  $\mu$ moles  $m^{-2} s^{-1}$  [400–700 nm]) and the relative humidity > 70%. Embryogenesis was induced from callus by transferring callus to induction medium containing SH medium with 10  $\mu$ M 2,4-dichlorophenoxy-acetic acid (2,4-D) and 5  $\mu$ M kinetin. Cultures were incubated on this medium for 4 d and transferred to embryoid medium. Embryoid medium contained SH medium with 50 mM L-proline and 30 mM L-glutamine and no phytohormones.

## RESULTS

#### Selection of Self-Incompatible Plants

A total of 435 plants from BMP8 AC3 and 482 plants from W10 AC3 reached flowering and were self-pollinated in the first cycle. Plants with self-fertility > 1 seed floret<sup>-1</sup> were discarded. Eighteen plants from the BMP8 AC3 population (4.1%) and nine plants from the W10 AC3 population (1.9%) were determined to be male sterile and were also discarded. During the second and third evaluation periods, five plants of BMP8 AC3 (1.1%) and eight plants of W10 AC3 (1.7%) were subsequently determined to be female sterile and were also discarded.

Following three evaluations of the plants, 28 plants superior in one of the following traits were selected: (i) low number of seed floret<sup>-1</sup> pollinated when selfed, (ii) high number of seeds floret<sup>-1</sup> when pollinated from a foreign alfalfa population, (iii) highly viable pollen, or (iv) high number of seed floret<sup>-1</sup> when used to pollinate a male-sterile clone (Table 1).

The percentage of hybrid plants formed when the self-incompatibles were crossed with the red-root clone was 98.6% (2135 red-root seedlings out of 2166 total seedlings). The red-root clone produced 107 red-root seedling (92.2%) and nine white-root seedlings (7.8%) when selfed, significantly fewer red-root seedlings than expected based on the other crosses made. Two hundred seventy four red-root seedlings were obtained from 277 total seedlings (98.9%) when pollen from the red-root clone was used to pollinate a male-sterile plant. Self-fertile plants (selections from 'Arc') produced 101 red-root seedlings of 130 total seedlings (77.7%) when crossed to the red-root clone. Thus, there appeared not to be a stimulation of the self-incompatible plants to accept their own pollen when in the presence of foreign pollen. This also demonstrated the significant reduction of selfing (1.4% vs. 22.3%) when self-incompatible plants are used in crosses.

#### Evaluation of the Half-Sib Progenies

Yields of half-sib families from two plants (168 and 189) from the parent population BMP8 AC3 were significantly ( $P < 0.05$ ) greater than the yield of the parent population (Table 2) indicating that these two plants combined well with the other self-incompatible plants. None of the half-sib lines tracing to W10 AC3

Table 1. Average number of seed floret<sup>-1</sup> produced when self-incompatible alfalfa plants were selfed, pollinated with foreign pollen, or crossed to a male-sterile plant.

Plant†	Self pollinated	Received foreign pollen	Crossed to male sterile	Pollen viability‡	
				seed floret <sup>-1</sup>	%
10§	0.15	2.8	2.9		44
23	0.20	5.7	1.7		48
45§	0.07	2.8	6.1		50
65	0.04	1.3	1.6		72
71§	0.03	4.7	1.3		51
82§	0.08	5.9	1.8		50
99	0.05	3.7	1.4		46
156	0.09	3.7	2.2		32
159§	0.07	3.0	3.1		77
165	0.40	2.6	2.9		45
168	0.18	3.0	1.6		40
189	0.19	4.4	2.2		53
190	0.05	2.4	1.7		45
203§	0.08	3.3	2.1		50
536§	0.01	3.6	2.8		63
587§	0.02	2.7	3.7		31
589§	0.03	2.4	2.8		58
598§	0.05	4.2	5.6		67
625	0.35	1.8	2.2		28
710§	0.01	1.9	3.0		42
740§	0.01	1.9	3.0		50
745	0.36	1.7	3.6		35
803	0.15	1.4	3.6		68
836	0.05	1.4	3.7		15
881	0.71	3.0	3.1		57
894	0.37	1.9	4.7		32
968	0.43	2.1	3.2		41
980	0.01	2.2	1.0		40
BMP8 AC3	1.14	3.5	2.3		50
W10 AC3	1.35	2.2	3.3		45

† Plants numbered 10 to 203 were selected from BMP8 AC3 and those numbered 536 to 980 were selected from W10 AC3.

‡ Determined by counting the number of pollen grains stained with cotton blue per 1000 pollen grains.

§ One of the 12 elite self-incompatibles.

differed significantly ( $P > 0.05$ ) from the parent population (Table 2).

Twelve elite self-incompatible plants were subse-

**Table 2.** Mean fresh weight of the half-sib progeny from self-incompatible (SI) alfalfa plants selected from populations W10 AC3 and BMP8 AC3.

SI plant†	Fresh wt.
	g
168	48.8
189	47.7
165	44.3
194	42.6
745	42.4
45	41.3
156	41.1
625	40.5
536	40.1
740	39.8
598	39.5
82	39.4
203	39.1
99	39.0
190	38.9
159	38.3
589	37.0
803	36.8
23	36.6
894	36.3
71	35.6
65	35.1
710	34.6
881	34.3
836	33.6
980	33.4
968	29.8
10	27.9
W10 AC3	36.1
BMP8 AC3	35.2
LSD (0.05)	10.5

† Self-incompatible plants numbered 10 to 203 were selected from BMP8 AC3 and those numbered 536 to 980 were selected from W10 AC3.

quently selected based on fertility and pollen viability. These lines produced (i)  $<0.15$  seed floret<sup>-1</sup> when self-pollinated, (ii)  $>30\%$  viable pollen, and (iii)  $>3$  seed floret<sup>-1</sup> when foreign pollen was applied to the self-incompatible or when the pollen from the self-incompatible was used as the pollen parent (Table 1).

#### Pollen Tube Growth Observations

Observations of pollen tubes of the 12 elite self-incompatible plants indicated that the mechanisms controlling self-incompatibility were associated with three types of prezygotic barriers: (i) pollen grains that did not germinate and penetrate the stigma, (ii) pollen tubes that penetrated the stigma did not grow through the style and enter the locule, and (iii) pollen tubes that grew near the ovules either formed a bulbous structure and terminated, formed several branches, or continued to grow past all of the ovules (Table 3). Frequently the pollen tubes formed multiple loops adjacent to an ovule, but did not enter the ovule. Prezygotic hybridization barriers were not complete, however. Also, these barriers did not account for all of the self-sterility in the self-incompatible plants, as the seeds per floret pollinated was usually less than the observed percent pollen tubes that reached the ovules (Table 3). Studies of the growth of foreign pollen in the pistils of the elite self-incompatible plants and the growth of pollen from these plants in the pistils of a male-sterile line showed a normal progression of pollen germination, pollen tube penetration of the stigma, growth through the style, and the termination of growth at the ovule. Some of the pollen tubes encountered the same prezygotic hybridization barriers as those described when selfing occurred; however, the

**Table 3.** Observations of pollen tube growth in elite self-incompatible alfalfa plants upon selfing and outcrossing.

Plant†	Ovules ovary <sup>-1</sup>	Pollen germinated	Tubes in style	Tubes in locule	Ovules reached	Seed floret <sup>-1</sup>	Ovules developed seed <sup>-1</sup>
		no.			%	no.	%
Self-pollinated							
10	10.4	11.2	4.9	4.5	0.23	0.15	1.44
45	10.3	9.9	5.3	3.8	1.59	0.07	0.68
71	10.5	9.2	3.3	2.5	0.23	0.03	0.29
82	11.0	11.7	6.5	5.9	1.01	0.08	0.73
159	11.2	4.9	2.1	1.5	0.43	0.07	0.63
203	9.9	5.8	3.2	2.8	4.60	0.08	0.81
536	10.1	9.4	5.2	5.1	0.73	0.01	0.09
587	10.1	10.2	4.0	3.7	0.17	0.02	0.20
589	9.3	27.1	17.8	11.2	4.75	0.03	0.32
598	9.1	6.6	4.6	3.0	0.14	0.03	0.33
710	9.9	5.4	3.1	2.4	0.00	0.01	0.10
740	9.2	9.2	6.3	5.9	0.02	0.10	1.09
Cross-pollinated							
10	10.0	20.1	19.6	19.6	20.6	2.79	27.90
45	9.7	27.6	27.5	27.5	43.0	2.79	28.80
71	10.4	18.3	15.7	15.7	36.3	4.71	45.30
82	11.0	20.8	20.7	20.7	54.5	5.95	54.10
159	9.8	5.8	4.4	4.4	8.4	3.00	30.61
203	9.5	45.8	45.5	45.5	39.8	3.28	34.50
536	9.8	20.7	16.9	16.9	22.4	3.56	36.33
587	9.5	22.5	21.8	21.7	31.7	2.68	28.20
589	9.7	24.5	17.5	17.4	44.6	2.40	24.70
598	9.7	6.3	3.4	3.4	13.9	4.20	43.30
710	9.1	8.3	4.2	4.2	13.1	1.94	21.31
740	8.4	17.1	8.4	8.3	18.1	1.93	22.98

† Plants numbered 10 to 203 were selected from BMP8 AC3 and those numbered 536 to 980 were selected from W10 AC3.

number of potential male gametes entering the locule was greater (Table 3).

#### *Production of Somatic Embryos*

Somatic embryos were produced from the callus cultures from all 12 elite self-incompatible plants. The cultures required from 30 to 60 d in the regeneration medium for somatic embryos to form. Plants 45, 82, 203, and 589 developed embryos in the shortest time (30 d), and plants 10 and 710 required >60 d before embryos were detected.

### DISCUSSION

Screening of two well-adapted multiple pest-resistant alfalfa populations yielded 3.0% male-sterile, 1.5% female-sterile, and 3.1% self-incompatible plants. Male sterility in alfalfa has been described as either nuclear (Childers and McLennan, 1960) or cytoplasmically controlled (Davis and Greenblatt, 1967). Female sterility was first reported by Childers (1960). Bingham and Hawkins-Pfeiffer (1984) determined that female sterility, caused by the incomplete development of the integuments, was controlled by a single recessive gene. Self-incompatibility in alfalfa was first described by Piper et al. (1914). Since that time, many alfalfa researchers have recognized self-incompatibility (reviewed by Viands et al., 1988). The mechanisms that result in self-incompatibility are not clearly understood and apparently are complex. Self-incompatibility in alfalfa has been characterized by (i) pollen-stigma interactions, typical of many sporophytic systems of control (Bolton and Fryer, 1937), (ii) pollen tube-style interactions, typical of most gametophytic controlled systems (Sayers and Murphy, 1966), and (iii) pollen tube-ovule interactions within the locule (Brink and Cooper, 1940). All of these phenomena were observed in the self-incompatible plants observed in this study.

Mulcahy and Mulcahy (1983) reexamined the conventional hypothesis of gametophytic self-incompatibility, based on the hypothesis that multiallelic incompatibility loci containing *S* alleles conditioning self-incompatibility were involved in the pollen-style interactions through the positive inhibition of incompatible pollen tubes. Their alternative hypothesis incorporates the concept of many closely linked loci (*supergene*) and complementary pollen-style interactions. However, they go on to state that "in some cases it may be appropriate to abandon the concept of incompatibility genes altogether and, instead, describe some angiosperm species which are unable to set seed after self-pollination, not as being self-incompatible, but rather as possessing too many deleterious recessive alleles to allow self-fertility." Cooper and Brink (1940) and Busbice (1968) have stated that inbreeding in alfalfa can cause the accumulation of too many deleterious recessive alleles to allow high levels of self seed production. Nevertheless, Sayers and Murphy (1966) observed, as we did in this study, that the same prezygotic hybridization barriers occur when alfalfa is outcrossed to an unrelated population, although at a much lower frequency. The same hybridization barriers were observed by Sangduen et al. (1983) and Bau-

chan and Elgin (1986) when observing pollen tube growth in interspecific hybridizations between *M. sativa* L. and *M. scutellata* (L.) Mill. Thus, inbreeding cannot account completely for the self-incompatibility phenomenon.

Regardless of the mechanisms involved in self-incompatibility, we have been able to identify self-incompatible plants that will produce 98.6% hybrid plants when hand pollinated. Preliminary yield trials conducted in a growth chamber of half-sib progenies from the intercrossing of self-incompatible plants indicate that certain combinations of self-incompatible plants exist that will yield hybrid progeny with increased forage production potential. Kehr (1976) and Veronesi and Lorenzetti (1983) concluded from their studies that if stands were adequate, a field composed of 75% hybrid plants was sufficient for maximizing forage productivity.

Many questions remain to be answered concerning the application of self-incompatibility to produce alfalfa cultivars. However, with the ability of self-incompatible clones to produce somatic embryos, coupled with the advancing technology in the use of artificial seeds to propagate elite germplasm in production fields; self-incompatibility in alfalfa may provide a mechanism for successfully producing high yielding hybrid alfalfa cultivars.

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