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INTERSPECIFIC POLLEN COMPETITION AS A REPRODUCTIVE BARRIER BETWEEN SYMPATRIC SPECIES OF *HELIANTHUS* (ASTERACEAE)[1]

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Artificial crosses between *Helianthus annuus* and *H. petiolaris* using 1:9, 1:1, and 9:1 mixtures of intraspecific : interspecific pollen were conducted to determine the role of interspecific pollen competition as a reproductive barrier in *Helianthus*. Of 1,245 achenes analyzed from the pollen competition experiments, only 49 were hybrids. The number of hybrids observed was significantly less than expectations for all three pollen mixtures, regardless of the identity of maternal parent ($P < 0.01$). Stigma age and pollen ratio had no significant impact on hybrid frequency. However, hybrids were significantly more frequent with *H. annuus* than with *H. petiolaris* as the maternal parent ($P < 0.01$). Analysis of pollen tube growth rates revealed no differences in the rate of growth of intraspecific vs. interspecific pollen. Likewise, pollinations with either intraspecific or interspecific pollen or with different pollen ratios did not affect the percentage of filled achenes. Thus, the mechanism responsible for selective fertilization by intraspecific pollen in mixed pollen loads remains unclear. Nonetheless, these findings suggest that interspecific pollen competition plays an important role in controlling the formation of hybrids between *H. annuus* and *H. petiolaris* and may partially account for patterns or differential cytoplasmic vs. nuclear introgression in *Helianthus*.

The possibility that selective fertilization by intraspecific pollen in mixed pollen loads might function as a reproductive isolating barrier was first suggested by Darwin (1859, p. 85) who noted that ". . . a plant's own pollen is always prepotent over foreign pollen." This observation has been shown to hold true in species pairs from a variety of plant groups including *Datura* (Buchholz, Williams, and Blakeslee, 1935), *Haplopappus* (Smith, 1968, 1970), perennial *Helianthus* species (Heiser et al., 1969), *Mimulus* (Kiang and Hamrick, 1978), and *Iris* (Arnold, Hamrick, and Bennett, 1993; Carney, Cruzan, and Arnold, 1994); although in *Mimulus* the prepotency of conspecific pollen was only observed with *M. guttatus* as the seed parent. Nonetheless, it seems likely that interspecific pollen competition plays a major role in controlling the formation of hybrids in many plant groups and thus should be considered in explanations of the occurrence, frequency, and geographic distribution of different genealogical classes of hybrids.

In *Helianthus,* interspecific pollen competition has been documented in crosses involving several perennial species, and perhaps accounts for the paucity of hybrids among sympatric, interfertile, perennial sunflower species (Heiser et al., 1969). In contrast, hybridization is common among the annual sunflowers of *H.* sect. *Helianthus,* and species integrity is thought to be maintained by strong chromosomal sterility barriers, which produce semisterility in hybrids (Heiser et al., 1969; Chandler, Jan, and Beard, 1986). Nonetheless, the possibility of interspecific pollen competition in crosses among species of sect. *Helianthus* has not been tested experimentally. Moreover, because interspecific pollen competition can control the identity of the maternal parent of hybrids, this phenomenon may also provide a possible explanation for patterns of differential cytoplasmic vs. nuclear introgression observed in several mosaic hybrid zones among the annual sunflowers.

This paper reports the results of a series of experiments designed to determine the occurrence and strength of interspecific pollen competition as a reproductive barrier between two annual sunflower species: *H. annuus* and *H. petiolaris.* In addition, we present data regarding the mechanism by which pollen competition leads to selective fertilization by intraspecific pollen in mixed pollen loads. Finally, we discuss the implications of these data for understanding patterns of hybridization and introgression in *Helianthus.*

Helianthus annuus, the common wild sunflower, is geographically the most widespread sunflower in North America and exhibits great morphological and habitat variation. The species tends to be weedy and is nearly always found in habitats that have been disturbed by humans. It is found in low and high rainfall areas from sea level to 3,000 m and flowers July through October. Attempts to describe some of that variation have been made by Heiser (1954) and Heiser et al. (1969), but no formal names have been proposed that adequately classify all its variants (Seiler and Rieseberg, in press). Results from extensive intraspecific crossing experiments (Heiser, 1954) indicate that the species is uniform cytologically; i.e., fertile plants were obtained from all crosses.

Helianthus petiolaris includes two widespread subspecies, subsp. *petiolaris* and subsp. *fallax* Heiser, which overlap geographically. Subspecies *petiolaris* is primarily found in the midwestern United States, whereas subsp. *fallax* occurs in the southwestern United States. Cyto-

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genetic studies have revealed three cytological races in *H. petiolaris* (Heiser, 1961; Heiser et al., 1969). One race consists of a single population of subsp. *petiolaris* from Minnesota, the second race includes subsp. *fallax* and several populations of subsp. *petiolaris,* and the third includes the rest of subsp. *petiolaris* (Heiser, 1961). The populations used for the interspecific pollen competition experiments are part of the third race.

Helianthus annuus and *Helianthus petiolaris* are easily distinguished by a number of morphological, chromosomal, and molecular features (Heiser et al., 1969; Chandler, Jan, and Beard, 1986; Rieseberg, Carter, and Zona, 1990). They occur in phylogenetically divergent clades (Schilling and Heiser, 1981; Rieseberg, 1991) and have different ecological requirements. In general, *H. annuus* is restricted to heavy soils and *H. petiolaris* to dry, sandy soils. Nevertheless, the two species grow together in a variety of locations and form a mosaic hybrid zone that extends over most of the central region of the United States. Meiotic analyses indicate that the two species have the same number of chromosomes ($n = 17$), but are quite different in terms of chromosome structure (Heiser, 1947; Whelan, 1979; Ferriera, 1980; Chandler, Jan, and Beard, 1986), with a wide range of multivalent configurations observed at meiosis that apparently correspond to two inversions and several translocations. Pollen viabilities in the F_1 hybrids are usually less than 10% (Heiser, 1947; Chandler, Jan, and Beard, 1986), and seed set is less than 1%. The percentages of good pollen in F_2 plants or BC_1 plants are highly variable, ranging from 13% to 97% (Heiser, 1947).

MATERIALS AND METHODS

Plant materials—Twenty plants from each of the following four accessions were grown in the greenhouse and used for experimental manipulations: NMS89 (a cultivar of *H. annuus* which segregates for nuclear male sterility); A101 (wild *H. annuus* collected 1 mi W. of MacDole, CA on Hwy. 97); P1224 (*H. petiolaris* subsp. *petiolaris* collected 0.3 mi. N. of the Canadian River, OK on I-44); and P1234 (*H. petiolaris* subsp. *petiolaris* collected 2 mi. S. of Muscatine, IA on Hwy. 61). Both cultivated and wild accessions were included in the study for several reasons. First, we were interested in the potential for hybridization and introgression between domesticated and wild sunflowers. Second, the large size of the cultivar inflorescence simplified experiments to study the effects of stigma age on interspecific pollen competition.

Pollen competition experiments—Because the plants were either self-incompatible or nuclear male sterile, it was not necessary to emasculate flowers prior to pollinations. For each of the pollination treatments, pollen was collected from newly open stamens representing five to ten plants from each accession. Pollen mixtures were then prepared by weight and applied with a small paintbrush to the inflorescences of two or more plants from each accession. All pollen applications were in excess of that needed to generate full seed set. The following pollen ratios were used: 1) 100% intraspecific pollen; 2) 90% intraspecific: 10% interspecific pollen; 3) 50% intraspecific: 50% interspecific pollen; 4) 10% intraspecific: 90% interspecific pollen; and 5) 100% interspecific pollen. In addition, to determine if stigma age affected interspecific pollen competition, the number of days between stigma appearance and pollen application was recorded. The frequency of filled achenes also was recorded for each experiment. Neff and Simpson (1990) demonstrate a well-defined temporal pattern to flowering in wild *H. annuus,* suggesting that not only the age of the stigma but also the time of day when the pollinations were performed might be significant. Thus, all pollinations were conducted in late morning.

Genotypes of parental plants and progeny were assayed using three loci: *Pgi2, Adh1,* and *Adh2.* Although *H. annuus* and *H. petiolaris* can usually be differentiated on the basis of *Pgi2* alone (Rieseberg, Carter, and Zona, 1990), variation at ADH was assayed as well to provide additional confirmation of progeny genotypes. Sample preparation and electrophoresis of PGI and ADH followed the sunflower isozyme protocols of Rieseberg, Soltis, and Soltis (1988).

Progeny genotype ratios were compared to pollen mixture ratios using chi-square analysis. The effects of maternal species, stigma age, and pollen ratio on arcsine-square root-transformed progeny genotype frequencies were analyzed using three-way analysis of variance (MGLH/GLM, SYSTAT Statistics, 1989). Likewise, a two-way analysis of variance was used to test for effects of maternal species and pollen ratio on the arcsine square-root transformation of the frequency of filled achenes.

Pollen tube growth rate experiments—*Helianthus* styles are 1–1.5 cm in length and have two branches. Thus, two types of pollen can be applied to the same style (one type to each branch), and within 2–3 hours sufficient pollen tube development will have occurred to allow comparison of pollen tube growth rates. In this experiment, pollen from accessions A101 and P1234 was applied to 25 styles of each species. After 2.5 hours, the styles were harvested and softened in 5 M NaOH overnight, rinsed in distilled water, and stained overnight in 0.1% aniline blue/0.1 N K_3PO_4. Observations of pollen tubes were made with an epifluorescence microscope. The lengths of a minimum of ten chosen pollen tubes from each style were measured with an ocular micrometer, and the means were analyzed using one-way analysis of variance (MGLH/GLM, SYSTAT Statistics, 1989). Typically, only ten to 15 discrete pollen tubes were observed on each side of the style, so all discrete pollen tubes were measured. Because the pollen tubes stained faintly between callose plugs, the actual measurements were from the center of the pollen clump on the style branch to the last observable callose plug. Thus, the use of the phrase "pollen tube growth rate" in this paper may be somewhat of a misnomer since the amount of extension of the pollen tube from the last callose plug is unknown.

In addition to direct observations of pollen tube growth, we varied the timing of interspecific and intraspecific pollen applications and assayed the resulting achene genotypes as described above. Specifically, pollen from *H. annuus* was applied to styles of *H. petiolaris* 15 minutes and 30 minutes prior to the application of *H. petiolaris* pollen to determine whether allowing *H. annuus* pollen a "head start" over *H. petiolaris* pollen might lead to an

TABLE 1. Genotype frequencies of *Helianthus* progenies from different interspecific pollen mixtures. NMS89 and A101 are *H. annuus* accession numbers, whereas P1224 and P1235 are *H. petiolaris* accession numbers.

Female	Male	% Hybrids	*N*	χ^2	% Filled achenes
		H. annuus			
NMS89	NMS89 vs. P1224				
	1:0	NA[a]	NA	NA	88.4
	1:9	7.9	239	1789***	89.6
	1:1	3.8	201	178***	87.1
	9:1	2.0	49	3.44	20.2
	0:1	NA	NA	NA	91.7
A101	A101 vs. P1235				
	1:0	NA	NA	NA	85.3
	1:9	9.7	72	516***	83.3
	1:1	2.0	50	18***	90.1
	9:1	3.6	58	2.77	86.4
	0:1	NA	NA	NA	85.9
		H. petiolaris			
P1224	P1224 vs. NMS89				
	1:0	NA	NA	NA	92.4
	1:9	0.0	173	1557***	87.1
	1:1	0.8	127	123***	75.1
	9:1	0.0	118	13.1***	90.3
	0:1	NA	NA	NA	90.7
P1235	P1235 vs. A101				
	1:0	NA	NA	NA	82.0
	1:9	2.0	49	421***	88.1
	1:1	0.0	50	50***	85.0
	9:1	0.0	51	5.7*	87.9
	0:1	NA	NA	NA	87.4

[a] Data not available.
***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

TABLE 2. Three-way analysis of variance of effects of the identity of maternal species, stigma age, and pollen ratio on progeny genotype frequencies.

Source of Variation	df	Sum of squares	*F*	*P*
Maternal species	1	0.1282	21.3724	0.0099
Pollen ratio	2	0.0300	2.4969	0.1978
Stigma age	2	0.0023	0.1914	0.8330
Maternal species × Pollen ratio	2	0.0325	2.7018	0.1802
Maternal species × Stigma age	2	0.0121	1.0114	0.4411
Pollen ratio × Stigma age	4	0.0260	1.0836	0.4699
Error	4	0.0240		

increased frequency of hybrids. The rationale for this experiment was that small differences in pollen tube growth rates not observable by fluorescence microscopy might be detectable in these pollen timing experiments.

RESULTS

Seed set—Pollinations with interspecific pollen only resulted in percentages of filled achenes of greater than 80% regardless of the maternal parent (Table 1). Similarly high percentages of seed set were observed for pollinations with intraspecific pollen only or with different ratios of intra- and interspecific pollen (Table 1). The only notable exception to these uniformly high seed set percentages involved one treatment with the cultivar NMS89 as the maternal parent, where a 9:1 pollen ratio yielded only 20.2% good seed. Unfortunately, we have no explanation for the high frequency of ovule abortion in this instance. Nonetheless, a two-way ANOVA indicated that the identity of the maternal species and pollen ratio had no significant effect on the percentage of filled achenes.

Pollen competition experiments—Of 1,245 achenes analyzed from the pollen competition experiment, only 49 were hybrids (Table 1). Of the hybrids, 47 were generated with *H. annuus* as the maternal parent and only two with *H. petiolaris* as the maternal parent. The number of hybrids observed was significantly less than expectations based on the *absence* of pollen competition for both the 50% intraspecific: interspecific pollen mixture and the 10% intraspecific: 90% interspecific pollen mixture, regardless of the identity of maternal species ($P < 0.001$; Table 1). Likewise, the number of hybrids was significantly lower than expectations for progenies from the 90% intraspecific: 10% interspecific pollen mixture ($P < 0.001$ and $P < 0.05$, respectively; Table 1), with *H. petiolaris* as the maternal species. However, genotype ratios for progenies with *H. annuus* as the maternal species did not differ significantly from the expected 9:1 (parental : hybrid) ratio.

The three-way ANOVA indicated that the identity of the maternal species had a significant effect on progeny genotype frequencies ($P < 0.01$; Table 2); hybrids were much more frequent with *H. annuus* as the maternal parent than with *H. petiolaris*. However, pollen ratio and stigma age had no significant effect on progeny genotype frequencies, nor were there significant interactions among the three factors.

Pollen tube growth rate experiments—No significant differences were observed in mean distance to the last observable callose plug after 2.5 hours regardless of maternal species (Fig. 1). However, analysis of 50 progeny from each of the two pollen timing experiments revealed no hybrids. One explanation for this result is that there are no differences in pollen tube growth rates between the two species and that some other mechanism is responsible for selective fertilization by intraspecific pollen. Alternatively, the use of callose plugs for measuring pollen tube growth rates may not be accurate.

DISCUSSION

The low frequency of hybrids from mixed pollen loads in *H. annuus* and *H. petiolaris* suggests that interspecific pollen competition has the potential to play a major role in limiting the formation of hybrids in natural populations of these species. However, for interspecific pollen competition to actually serve as an isolating barrier, pollen from both species must frequently co-occur on styles in sympatric or mixed populations of the two species. Because *H. annuus* and *H. petiolaris* overlap almost completely in flowering time and pollinators do not appear to discriminate between them (Rieseberg, unpublished data), the delivery of mixed pollen loads to individuals in sympatric populations seems likely. Thus, interspecific pollen competition may be a major component of repro-

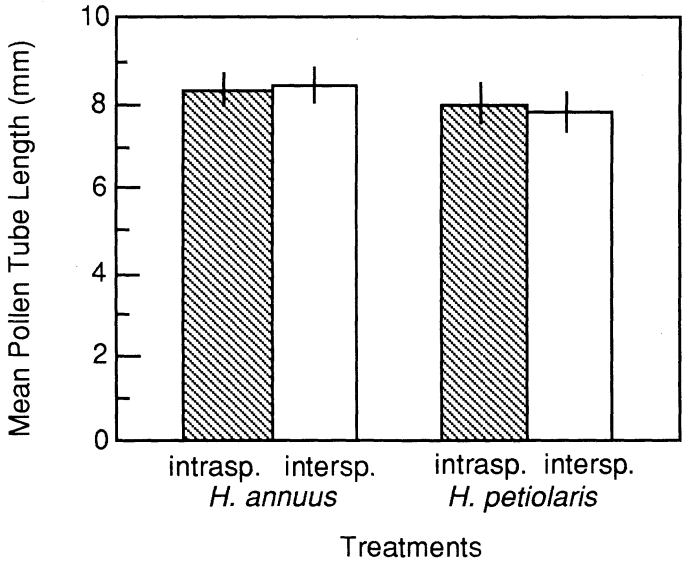

Fig. 1. Mean pollen-tube lengths of intraspecific and interspecific pollinations with *H. annuus* and *H. petiolaris* as maternal plants. Standard errors are indicated by vertical lines. Mean pollen tube lengths were not significantly different with either species as maternal parent.

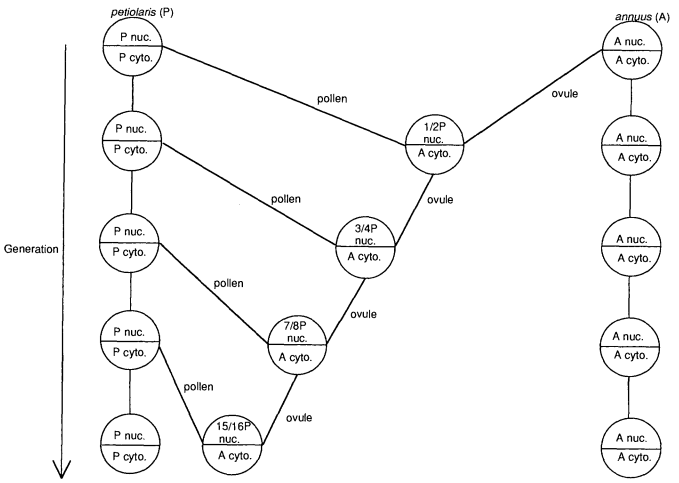

Fig. 2. Hypothetical scenario for cytoplasmic introgression in a population of *H. petiolaris* following the introduction of a single individual of *H. annuus*. Because of pollen competition, *H. annuus* serves as the maternal parent (ovule). Male sterility in first and later generation hybrids and backcrosses quickly leads to the production of plants that have the cytoplasm of *H. annuus*, but whose nuclear genes are predominantly those of *H. petiolaris*.

ductive isolation in both the annual and perennial sunflowers.

One possible consequence of interspecific pollen competition noted by Arnold, Hamrick, and Bennett (1993) is that hybrid formation is likely to be relatively rare and often restricted to populations where flowering individuals of one species are in a quantitative minority. This would create a situation where the pollen load delivered to the minority species would consist primarily or entirely of foreign pollen and thus be more likely to result in the production of hybrids. This "minority" hypothesis has implications for explaining patterns of differential cytoplasmic vs. nuclear introgression reported in many plant groups (reviewed in Rieseberg and Soltis, 1991) because minority species will serve as the maternal parent of hybrids far more frequently than the majority species. Male sterility in hybrids and introgressants could quickly lead to the presence of individuals carrying the cytoplasm of the minority species and the nuclear genes of the majority species (Fig. 2). Continuing this scenario, individuals from the "hybrid founder population" could expand their geographic distribution, leading to the differential patterns of cytoplasmic vs. nuclear introgression observed in many plant hybrid zones, including several in *Helianthus* (reviewed in Rieseberg and Soltis, 1991). A potential problem with this scenario is the situation previously reported in *Iris*, where selective fertilization by intraspecific pollen is a major component of reproductive isolation (Arnold, Hamrick, and Bennett, 1993), but differential cytoplasmic/nuclear introgression has not been observed. Rather, introgression appears to be pollen-mediated (Arnold, Bennett, and Zimmer, 1990; Arnold, Buckner, and Robinson, 1991). However, *Iris* hybrids produce fertile pollen, whereas pollen generated by hybrids among the annual *Helianthus* species is highly sterile (Heiser et al., 1969; Chandler, Jan, and Beard, 1986), perhaps explaining the different patterns of introgression observed. Nonetheless, several alternative explanations for differential cytoplasmic vs. nuclear introgression in *Helianthus* that do not require interspecific pollen competition are discussed by Rieseberg, Choi, and Ham (1991) and Dorado, Rieseberg, and Arias (1992).

Another interesting result from the pollen competition experiments was that hybrids were much more frequent with *H. annuus* as the maternal parent than with *H. petiolaris*. This observation or "asymmetry" hypothesis may account for the fact that in five of six cases of cytoplasmic introgression observed in *Helianthus* in nature, *H. annuus* has served as the cytoplasmic donor (Rieseberg et al., 1991; Rieseberg, Choi, and Ham, 1991; Dorado, Rieseberg, and Arias, 1992). Moreover, two of these instances involved hybrids between *H. annuus* and *H. petiolaris*.

Although we had hoped to determine the actual mechanism responsible for selective fertilization by intraspecific pollen in mixed pollen loads, our results were inconclusive. No differences were observed in pollen tube growth rates as measured by mean distances to callose plugs. Possibly, we sampled the florets too early or too late to detect differences in pollen tube growth rates, or the use of callose plugs for measuring pollen tube growth rates may not be accurate. Alternatively, the rate at which the pollen tubes entered the micropyle may be different, as suggested by the pollen timing experiments. For example, selective fertilization by intraspecific pollen due to a mismatch of male and female gametophytic maturity periods resulting in pollen tube overgrowths has been observed in *Rhododendron* (Williams et al., 1986; Williams and Rouse, 1990). However, we have no evidence relevant to these hypotheses. Alternatively, the low frequency of hybrids may be due to hybrid ovule abortion, which has been suggested as an explanation for reduced hybrid seed formation in *Iris* (Carney, Cruzan, and Arnold, 1994).

However, this hypothesis seems unlikely in *Helianthus* because pollinations with interspecific pollen resulted in high percentages of filled achenes with either species as the maternal parent. Moreover, percentages of filled achenes in the pollen mixture experiments were not correlated with pollen ratios. A final possible explanation relates to the observation that foreign pollen on a stigma can lead to selfing in normally self-incompatible plants including *Helianthus* (C. Heiser, personal communication). However, the frequency of selfing and whether it occurs in crosses between *H. annuus* and *H. petiolaris* is unknown. Nonetheless, it is possible that the low frequency of hybrids, at least in situations where the pollen ratio is biased toward interspecific pollen, may be partly due to selfing.

In conclusion, the potential importance of selective fertilization by intraspecific pollen as an isolating barrier in plants appears to have been largely unappreciated until recently (Arnold, Hamrick, and Bennett, 1993; Arnold, 1994; although see Smith, 1968, 1970). This is surprising given the significance placed on this phenomenon by Darwin (1859) and its obvious relevance toward understanding the occurrence, frequency, and geographic distribution of different genealogical classes of hybrids. In sunflower, it may partially account for the occurrence of differential cytoplasmic vs. nuclear introgression and explain why *H. annuus* is the maternal parent of most hybrids generated among the annual sunflower species. In *Iris,* selective fertilization by intraspecific pollen appears to explain the rarity of F_1 hybrids relative to other hybrid classes (Arnold, Hamrick, and Bennett, 1993). Both studies suggest that hybrid formation may be rarer than originally thought and suggest the need for additional studies to assess the "universality" of this phenomenon in plants.

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