Interspecific pollen–pistil incongruity in *Salix*

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Pollination barriers that restrict hybridization between six *Salix* spp. (*S. amygdaloides* Anderss., *S. exigua* Nutt., and *S. lucida* Muhl., of subgenus *Salix*, and *S. discolor* Muhl., *S. eriocephala* Michx., and *S. petiolaris* Smith of subgenus *Vetrix*) and that are sympatric over much of their ranges in central North America were investigated through artificial cross pollination. Foreign species' pollen generally adhered to and germinated on the stigma. Pollen–pistil incongruity was most often expressed as reduced pollen tube growth rate, but the degree of incongruity was highly variable within and between different species combinations. Morphological abnormalities such as swollen, coiled, and undirected pollen tube growth were observed in some crosses but were not common. The stigma of *S. eriocephala* Michx. exhibited a particularly strong and characteristic inhibition of all foreign species' pollen tube growth. Despite the presence of some form of pollen–pistil incongruity in most interspecific crosses, pollen tube penetration of the ovule micropyle (fertilization) was successful in several species combinations.

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Des expériences de pollinisation croisée artificielle ont permis d'étudier les barrières qui nuisent à l'hybridation de six espèces du genre *Salix* (*S. amygdaloides* Anderss., *S. exigua* Nutt. et *S. lucida* Muhl., du sous-genre *Salix*, et *S. discolor* Muhl., *S. eriocephala* Michx. et *S. petiolaris* Smith, du sous-genre *Vetrix*) dont la répartition dans le centre de l'Amérique du Nord est à peu près sympatrique. En général, le pollen des espèces étrangères adhère au stigmate de la plante et y germe. L'incompatibilité du pollen et du pistil se reflète le plus souvent par un ralentissement de la croissance du tube pollinique, mais l'importance de cette incompatibilité varie considérablement pour chaque combinaison et entre les combinaisons. Des anomalies morphologiques (renflement, enroulement ou désorientation du tube pollinique) ont été observées dans certains croisements, sans toutefois être fréquentes. Le stigmate du *S. eriocephala* Michx. a démontré une inhibition particulièrement forte et caractéristique à la croissance du tube pollinique de toutes les autres espèces. Malgré une certaine incompatibilité entre le pollen et les pistils de la plupart des croisements interspécifiques, la pénétration du micropyle de l'ovule par le tube pollinique (fécondation) a effectivement eu lieu dans plusieurs combinaisons entre espèces.

Introduction

The rejection of foreign species' pollen by pistillate plants represents an important breeding barrier to interspecific hybridization in several tree species (Guries and Stettler 1976; Levin 1978; Stettler *et al.* 1980; Ager and Guries 1982; Gaget *et al.* 1984; Bob *et al.* 1986). Hogenboom (1973) referred to nonfunctioning interspecific pollen–pistil relationships as "incongruity": a reproductive barrier arising as a by-product of evolutionary divergence among populations or species. The concept of incongruity emphasizes the whole complex of metabolic and physiological interactions that define a successful pollen–pistil relationship (Stanley and Linskens 1974; Knox 1984) by recognizing that any genetic incapacity to coordinate pollen germination, penetration, and tube growth can prevent successful fertilization.

Abnormalities of pollen tube growth can be expressed as reduced growth rate, pollen tube swelling and coiling, or undirected pollen tube growth (Hagman 1975; Kho *et al.* 1980; Summers and Grun 1981; Williams *et al.* 1982). The degree of incongruity depends not only on the species crossed but also on the direction of the cross (Hogenboom 1972; Kho *et al.* 1980) and the specific combining ability of the genotypes within a species combination (Burson and Young 1983). Ostrolucka (1979) observed that foreign species' pollen adhered to and germinated on the stigma surface in crosses between several European *Salix* spp., but to my knowledge no other work has been done on pollen–pistil relationships in willows.

The strength of reproductive barriers between closely related species determines the amount of interspecific gene flow via introgression, and the success of artificial hybridization for breeding purposes. Willows provide a unique opportunity to investigate reproductive barriers because it is not uncommon to find five or six different willow species occupying the same wetland habitat (Mosseler 1987); hence reproductive barriers are of particular interest in willows because of their potential evolutionary impact through hybridization (Nilson 1918). The objectives of this investigation were (*i*) to describe pollen–pistil relationships between some of the major willow species of North America; (*ii*) to assess the importance of pollen–pistil incongruity as a barrier to gene flow and breeding; (*iii*) to clarify phylogenetic relationships based on species crossability; and (*iv*) to document flower longevity and other aspects of the reproductive biology of willows. The *Salix* species studied have been classified into two subgenera by Dorn (1976) as follows: *S. amygdaloides* Anderss., *S. exigua* Nutt., and *S. lucida* Muhl. into subgenus *Salix*; *S. discolor* Muhl., *S. eriocephala* Michx., and *S. petiolaris* Anderss. into subgenus *Vetrix*.

Materials and methods

Controlled pollinations

Dormant flower branches (approximately 50 cm in length) were cut from clonally propagated ortets originating from natural populations in southern Ontario, Canada (Mosseler 1987). The best success with unrooted stem cuttings in controlled pollinations was achieved when shorter (less than 20 cm in length) flower branches were used, particularly with the precocious flowering, difficult to root species like *S. discolor* and *S. petiolaris*. Flower branches were potted in a 2:1 PROMIX B : washed-sand soil medium and placed

TABLE 1. The expected mean squares for ANOVA of number of seeds set per capsule in flowers pollinated on different days following the onset of receptivity

Source of variation	df	Expected mean squares	Denominator for F test
Days	$(x-1)$	$\sigma_\epsilon^2 + 15\sigma_C^2 + 15c\sigma_D^2$	MS_C
Crosses	$x(y-1)$	$\sigma_\epsilon^2 + 15\sigma_C^2$	MS_ϵ
Error	$14xy$	σ_ϵ^2	

NOTE: The number of days (x) of the receptive period varied between 2 and 6, and the number of crosses per species (y) varied from 6 to 36, depending on species.

in a hydroponic irrigation system. Male and female plants were grown in separate compartments to avoid pollen contamination. Natural lighting was supplemented with sodium vapour lights to maintain a 16-h photoperiod.

Male catkins were removed at anthesis and fresh pollen was deposited directly onto the receptive flowers. Since the six species investigated have different seasonal flowering periods (Mosseler and Papadopol 1989), male and female flower branches from different species were forced to flower at the same time by potting each species in a predetermined sequence over a period of several weeks. In this manner, fresh pollen from different species was available as female flowers entered receptivity.

Pistillate parents from a particular species were pollinated on the same day between 07:00 and 09:30 in glasshouse compartments maintained at approximately 20 ± 5°C. Each male catkin was tested for pollen viability on an agar medium containing 0.6% (w/v) agar, 5.0% (w/v) sucrose, and 0.001% H_3BO_4, and pollen viability was assessed after 24 h using a dissecting microscope to determine the proportion of germinated pollen.

Flower receptivity

Each female flower was tagged to mark the onset of receptivity, the point at which the lobes of the stigma became fully reflexed. The length of the receptive period for each species was determined by making intraspecific crosses at regular intervals for 6 days after the onset of receptivity. At maturity, the number of seeds set per capsule was determined from 15 randomly selected capsules per catkin. A two-level nested ANOVA was computed with this data to test for differences between the days on which pollination was carried out (1 to 6 days after entering receptivity) and between different genotypic combinations within each species. The purpose of this ANOVA was to obtain a conservative estimate of the length of the receptive period based on the number of seeds set per capsule. The linear model describing this experiment was as follows:

$$Y_{ijk} = u + D_i + C_{j(i)} + \epsilon_{(ij)k}$$

where Y_{ijk} is the average number of seeds set in the kth flower capsule of the jth cross nested within the ith day of receptivity; u is the experimental mean number of seeds set; $C_{j(i)}$ is the effect of the jth female × male crossing combination nested within the ith day (D_i); and $\epsilon_{(ij)k}$ is the experimental error. This is a mixed model in which days were considered as a fixed factor and crosses were considered as random effects in calculating the expected mean squares (Table 1).

Fluorescence microscopy

The mating system used for controlled pollinations in the microscopic study of pollen–pistil interactions included two or three pistillate parents crossed in all combinations with two or three pollen parents. No pollen mixtures were used in these controlled crosses. A minimum of three flowers per catkin from each of three catkins per cross were harvested at 3, 6, 9, 12, 24, and 48 h after pollination.

The techniques used for preparation and staining of tissues for fluorescent microscopy were adapted from Martin (1959). Flowers were fixed in a 3:1 solution of absolute ethanol and glacial ascetic acid overnight and rinsed in 70% ethanol. The pistils were macerated in 20% NaOH for 4 to 6 h, depending on species requirements, and then rinsed in distilled water, cleared in full strength commercial bleach (Javex: 6% sodium hypochlorite) as recommended by O'Brien (1974) for up to 30 min, and rinsed again in distilled water. The cleared pistils were stained with 0.5% (w/v) aniline blue stain dissolved in a phosphate buffer to maintain a pH of 8.75. Slides were prepared as squash mounts and viewed under ultraviolet illumination from a high pressure mercury vapour light source in a Polyvar (Reichert Jung) microscope with a B1 barrier filter.

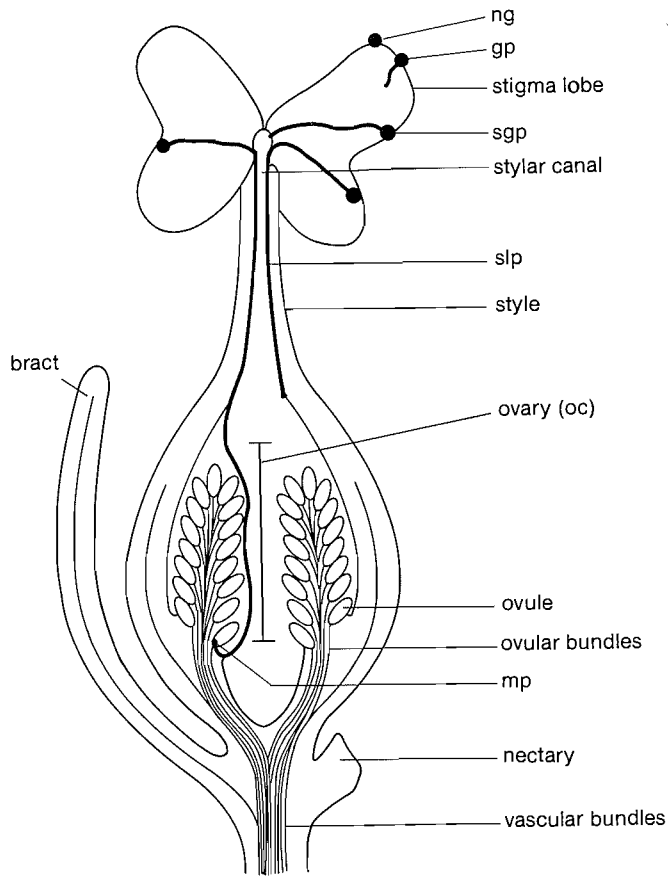


FIG. 1. Diagram of the *Salix* flower pistil showing the classification of pollen tube growth as follows: pollen grains adhering to stigma but failing to germinate (*ng*), pollen grains germinating but failing to grow over the stigma (*gp*), pollen tubes growing over the stigma (*sgp*), in the style (*slp*), into the ovarian cavity or locule (*oc*), and penetrating the ovule micropyle (*mp*).

Pollen tube growth was assigned to one of the following categories as described in Fig. 1: (*i*) no pollen grains observed on the stigma; (*ii*) pollen tube extension was less than 4 times the diameter of the pollen grain; (*iii*) pollen tube growth restricted to the stigma; (*iv*) pollen tubes extended into the upper half of the style; (*v*) pollen tubes extended into the lower half of the style; (*vi*) pollen tubes extended into the ovarian cavity or locule; and (*vii*) pollen tube penetration of the ovule micropyle.

Results and discussion

Female flower receptivity

Variations in flower longevity have ecological and genetic significance, yet there is little information in the botanical literature on this subject (Primack 1985). Among willow species, large variations in the length of the receptive period and the number of seeds set per capsule were evident (Table 2). Under glasshouse conditions, *S. lucida* suffered

TABLE 2. Average seed set per capsule (and range) and the success ratio (SR) of attempted intraspecific crosses on different days following the onset of receptivity

	S. amygdaloides		*S. discolor*		*S. eriocephala*		*S. exigua*		*S. lucida*		*S. petiolaris*	
Days	SR	Seed set	SR	Seed set	SR	Seed set	SR	Seed set	SR	Seed set	SR	Seed set
1	5/5	15.9 ± 0.53 (14.4–17.7)	4/4	10.3 ± 1.22 (7.7–12.5)	6/6	15.0 ± 0.67 (11.7–16.4)	5/5	24.4 ± 3.68 (15.0–35.6)	7/7	17.4 ± 1.08 (12.0–20.0)	6/6	3.2 ± 0.43 (1.9–5.0)
2	5/5	16.0 ± 1.40 (13.6–21.5)	4/4	9.0 ± 2.16 (6.7–15.5)	5/5	15.8 ± 0.70 (14.0–17.8)	6/6	23.0 ± 1.20 (9.9–27.3)	6/6	18.6 ± 1.07 (15.1–21.0)	3/6	3.6 ± 0.85 (1.9–4.5)
3	4/4	15.0 ± 0.76 (13.5–17.1)	2/6	9.1 ± 0.81 (8.5–9.7)	4/5	13.1 ± 1.74 (10.3–17.8)	5/6	16.9 ± 2.86 (5.7–21.5)	6/6	20.3 ± 0.74 (18.5–23.6)	1/4	3.9 ± 1.06
4	4/4	13.4 ± 0.40 (12.7–14.5)	2/4	4.6 ± 3.16 (2.3–6.8)	2/6	7.1 ± 1.27 (6.2–8.0)	5/6	15.2 ± 2.89 (7.3–24.5)	6/7	20.4 ± 0.57 (18.9–22.1)	1/5	3.0 ± 0.65
5	3/3	11.4 ± 0.72 (10.1–12.6)	1/5	6.7 ± 0.88	1/6	4.1 ± 0.82	1/5	15.9 ± 0.96	6/8	18.9 ± 1.81 (10.3–22.1)	0/5	
6	3/3	9.1 ± 1.98 (5.2–11.5)	1/6	11.6 ± 0.52	0/6		0/4		6/9	19.8 ± 0.96 (14.9–20.5)	0/5	

NOTE: Results are given as mean number of seeds set per flower capsule ± SE; the range in values is in parentheses.

no significant (at $P < 0.05$) loss of receptivity over the 6 days following the onset of receptivity, whereas most pistillate plants of *S. discolor* and *S. petiolaris* remained receptive for only 48 h (Tables 2 and 3). The appearance of receptivity proved to be deceptive in flowers of *S. discolor*, *S. eriocephala*, and *S. petiolaris*, in which flower stigmas maintained a receptive appearance for several days after they had lost receptivity. The highly significant differences (at $P > 0.001$) in number of seeds set between different intraspecific crossing combinations may indicate important differences in fecundity either between different females within a species or between different genotypic (female × male) combinations (Table 3). *In vitro* pollen germination tests were carried out for each controlled pollination attempted, but no important differences in pollen germination rate were evident. Unfortunately, the data set was too unbalanced to use ANOVA tests for the effects of the pistillate parent, the pollen parent, or female × male interactions on flower longevity or number of seeds set per capsule.

Salix amygdaloides, *S. lucida*, and *S. petiolaris* showed a large component of variance due to within-catkin differences (experimental error). Microscopic observations showed that artificially pollinated stigmas were usually laden with abundant pollen. Physiological differences or variation in receptivity between flower capsules may account for such a large within-catkin (between flowers) component of variation in seed set in certain species (Table 3). For purposes of the microscopic study of pollen–pistil relationships, a conservative estimate of the length of the receptive period for each species was as follows: *S. amygdaloides*, 4 days; *S. discolor*, 2 days; *S. eriocephala*, 3 days; *S. exigua*, 4 days; *S. lucida*, a minimum of 6 days; and *S. petiolaris*, 2 days.

In a related study, Mosseler (1987) found that the early flowering willows, *S. discolor*, *S. eriocephala*, and *S. petiolaris*, lacked the ecological and phenological reproductive barriers that would restrict or prevent natural hybridization. The shorter receptive periods that have been observed in these species may have an adaptive function in restricting natural hybridization. Since flower longevity can affect the degree of outcrossing (Primack 1985), the reduced fitness in interspecific willow hybrids observed by Mosseler (1987) could place natural selection pressures on flower longevity in hybridizing species to promote premating reproductive isolation.

Pollen viability

Pollen viability was high (above 70%) in all species when pollen was collected within 48 h of anthesis. A deterioration in viability was accompanied by changes in colour from bright yellow or orange yellow of freshly shed pollen to a bleached yellow. Male catkins could be stored under dessication at 3°C for several weeks without losing viability.

Pollination barriers to hybridization

Pollen–pistil incongruity was expressed as reduced pollen tube growth rate (Table 4; Figs. 3, 12, and 13) and various morphological abnormalities, such as swelling, coiling, and undirected pollen tube growth on the stigma (Figs. 2, 4, 5, and 6). These forms of incongruity have also been reported in interspecific crosses in the genera *Populus* (Guries and Stettler 1976, Stettler *et al.* 1980) and *Ulmus* (Ager and Guries 1982, Bob *et al.* 1986). In most interspecific *Salix* crosses, the only evidence of incongruity was reduced pollen tube growth rates over the stigma and inner wall of the style (Table 4); similar to observations made by Stettler *et al.* (1980) in *Populus*. Pollen tubes entering the style rarely showed any of the morphological abnormalities associated with stylar incongruity in other species (Williams *et al.* 1982).

Pollen tubes appeared to grow over the surface of the stigma and the inner wall of the style (Figs. 8, 11, and 12). The presence of intercellular penetration of female flower tissue could not be detected using fluorescence light microscopy. Pollen tubes were funnelled through a hollow style (canal) into the locule (Figs. 5 and 8). Large intrastylar differences were observed in pollen tube growth rates (Figs. 7 and 11): a demonstration of intense pollen competition for fertilization and the potential selective function of pollen–pistil interactions (Mulcahy 1971, 1979; Mulcahy and Mulcahy 1975; Marshall 1988). In all intraspecific crosses, the first pollen tubes entered the ovule micropyle between 12 and 24 h after pollination (h.a.p.) but the majority of fertilizations occurred between 24 and 48 h.a.p. Stigmas lose their receptivity shortly after pollination. This loss of receptivity became evident as a brown discoloration and by increased background fluorescence under the microscope.

In most crosses, foreign species' pollen adhered to and

germinated on the stigma surface (Table 4), suggesting that in *Salix*, the initial adhesion of foreign species' pollen to the stigma surface depended on nonspecific chemical interactions as outlined by Ferrari *et al.* (1985), rather than on the existence of the species-specific recognition proteins described by Hagman (1975) and Knox *et al.* (1972, 1976).

In *S. discolor*, pollen–pistil incongruity was expressed as inhibition of pollen tube growth upon germination (Fig. 2) and by impeded growth over the stigma and style in *S. discolor* × *S. eriocephala* crosses (Fig. 3). In only one crossing combination were pollen tubes observed in the locule and penetrating the ovule micropyle. A similar pattern of pollen tube growth inhibition was observed in *S. discolor* × *S. exigua* crosses (Fig. 4), in which pollen tubes generally ceased growth within 12 h of germination. In many interspecific crosses, the upper style represented an absolute barrier to pollen tube growth. All successful fertilizations between *S. discolor* × *S. exigua* and *S. discolor* × *S. eriocephala* involved the same pistillate parent (*S. discolor* clone 58), demonstrating the effect of the pistillate genotype in controlling the interspecific crossability relationship.

In *S. eriocephala*, all foreign species pollen tube growth was arrested on the stigma surface upon germination (Fig. 5). No foreign species' pollen was able to grow more than several diameters of the pollen grain over the stigma. The appearance of the short, swollen, and twisted foreign pollen tubes (Fig. 6) contrasted sharply with the appearance of pollen tubes in most other interspecific crosses, in which incongruity was usually demonstrated by reduced pollen tube growth rate (Figs. 3 and 12) without any morphological abnormalities evident in the pollen tubes. In several crosses, foreign species' pollen grains that had become lodged in the open, central canal (Fig. 5) of the style (thereby avoiding contact with the stigma surface) were able to germinate and develop normal pollen tubes capable of entering the locule. This phenomenon was observed in all interspecific crosses involving *S. eriocephala* as the pistillate parent, demonstrating that inhibition on the stigma surface was the primary barrier to fertilization. Penetration of the ovule micropyle was observed in crosses where *S. amygdaloides*, *S. discolor*, and *S. exigua* were the pollen parents. Hybrid seeds were brought to term in *S. eriocephala* × *discolor* and *S. eriocephela* × *exigua* crosses.

In *S. exigua*, the growth of *S. amygdaloides* pollen tubes was restricted to the stigma 48 h.a.p., and stigma papillae were often highly fluorescent. Those flowers in which pollen tubes were observed within the lower half of the style or within the locule came from only two of the seven crosses investigated. Fertilization (micropyle penetration) was observed in only one interspecific combination, but hybrid seeds were not recovered from this cross. Pollen–pistil incongruity in *S. exigua* × *S. lucida* crosses was expressed as an inhibition of pollen tube growth on the stigma surface. Only rarely were *S. lucida* pollen tubes able to grow more than several diameters of the pollen grain in length.

A fertilized ovule of *S. lucida* is shown in Fig. 9 shortly after fertilization, and the arrangement of ovules in the ovary is shown in Fig. 10. In *S. lucida*, pollen tube growth of *S. discolor*, *S. eriocephala* (Fig. 13), *S. exigua* (Fig. 12), and *S. petiolaris* was usually arrested in the upper style at 12 h.a.p. However, one pistillate parent of *S. lucida* (clone 32) was consistently more receptive to all foreign

TABLE 3. ANOVA of number of seeds set per capsule over the receptive period in *Salix* spp.

Source of variation	*S. amygdaloides*			*S. discolor*			*S. eriocephala*			*S. exigua*			*S. lucida*			*S. petiolaris*		
	df	MS	VC	df	MS	VC	df	MS	VC	df	MS	VC	df	MS	VC	df	MS	VC
Days	3	130ns	12	1	53ns	14	2	46ns	6	3	836ns	2	5	151ns	2	1	8ns	7
Crosses	12	53***	28	3	370***	66	9	91***	46	16	725***	78	30	112***	29	4	18***	38
Error	224	7	60	112	7	19	168	6	49	280	12	19	504	15	69	84	2	55

NOTE: ***, significant at $p < 0.001$; ns, not significant, $p > 0.05$; VC, variance component as a percentage of total variation.

TABLE 4. Location of the 10 longest pollen tubes in flower pistils at 12, 24, and 48 h after pollination

Cross combination	N^b	Time[c]	% of pistils with pollen tubes[a]							
			ABS	GER	STA	UST	LST	LOC	MP[d]	Total[e]
S. discolor ×										
S. discolor	6	12	8.4	10.8	54.2	26.5				83
		24		5.2		67.2	8.6	19.0	5	116
		48		0.9	3.8	10.4	12.3	72.6	42	106
S. eriocephala	8	12	8.8	31.2	40.0	20.0				80
		24	3.7	32.6	23.0	36.4	1.1	3.2	1	187
		48	3.7	29.5	11.0	42.1	2.6	11.0	12	189
S. exigua	5	12		37.7	20.8	26.4	15.1			53
		24	2.1	20.8	31.2	33.3	12.5			96
		48	1.9	13.1	34.6	34.6	11.2	4.7	2	107
S. petiolaris	4	12		38.2	41.2	20.6				34
		24	1.1	50.0	32.2	16.7				90
		48	3.0	21.2	12.1	63.7				99
S. eriocephala ×										
S. eriocephala	6	12	13.6			11.6	56.3	18.4		103
		24		9.6		4.4	7.0	78.9	22	114
		48	4.2					95.8	99	142
S. amygdaloides	6	12		100						131
		24	10.1	89.9						149
		48	7.8	92.2						128
S. discolor	6	12		100						81
		24	16.6	83.4						145
		48	10.8	89.2						158
S. exigua	3	12	29.3	70.7						41
		24	9.4	90.6						64
		48		100						57
S. lucida	6	12	30.2	69.8						96
		24	29.2	70.8						161
		48	18.5	81.5						124
S. petiolaris	3	12		100						52
		24		100						58
		48	8.3	91.7						48
S. exigua ×										
S. exigua	5	12			3.6	37.8	58.5			82
		24	4.5			17.9	9.0	68.6	16	67
		48	5.3			5.3	1.8	87.7	33	57
S. amygdaloides	7	12	9.3	66.9	16.9	6.8				118
		24	5.5	32.9	20.5	35.6	5.5			73
		48	8.1	31.4	27.9	18.6	1.2	12.8	3	86
S. lucida	5	12	12.1	87.9						91
		24	4.7	89.4	5.6					85
		48	2.8	97.2						72
S. lucida ×										
S. lucida	5	12				23.1	64.1	12.8		39
		24	1.9	3.8		9.6	30.8	53.8	7	52
		48	10.1	3.8		2.5	2.5	81.0	43	79
S. amygdaloides	6	12	3.4	17.9	2.6	70.1	6.0			117
		24	5.1	12.1	11.1	29.3	42.4			99
		48	6.9	4.2	2.8	50.0	25.0	11.1	4	72
S. discolor	4	12			6.9	90.3	2.8			72
		24	2.4	2.4	3.6	74.7	16.9			83
		48	1.8	7.1		76.8	14.3			56
S. eriocephala	3	12		8.0	22.0	70.0				50
		24	4.7	6.2	37.5	51.5				64
		48	7.4	18.5	7.4	66.7				54
S. exigua	9	12	12.6	9.5	11.4	66.4				158
		24	9.6	22.2	13.4	53.9	0.6			167
		48	10.5	2.8	9.1	72.7	4.2	0.7		143
S. petiolaris	2	12	23.7	13.2	13.2	50.0				38
		24	7.3	19.5	9.8	63.4				41
		48		6.7	16.7	76.7				30

TABLE 4 (*concluded*)

Cross combination	N^b	Time[c]	% of pistils with pollen tubes[a] ABS	GER	STA	UST	LST	LOC	MP[d]	Total[e]
S. petiolaris ×										
S. petiolaris	3	12		15.6		56.2	28.1	3.1		32
		24	2.5	10.0		5.0		82.5	4	40
		48		8.9				91.1	12	45
S. amygdaloides	8	12	46.5	53.5						99
		24	48.0	52.0						77
		48	61.2	38.3						115
S. discolor	5	12	12.5	87.5						72
		24		100						76
		48	8.6	89.6	1.7					58
S. eriocephala	5	12	3.6	46.4	32.1	17.8				56
		24		42.0	14.8	18.2	14.8	10.2		88
		48	7.4	36.8		5.9		50.0	12	68
S. exigua	6	12	19.4		5.6	66.7	8.3			36
		24	26.2		7.1	9.5	2.4	54.8	8	42
		48	13.9	8.3		2.8		75.0	36	72
S. lucida	8	12	50.4	49.6						125
		24	62.6	37.4						107
		48	78.7	21.3						108

[a]The percent of pistils observed with pollen absent from stigma (ABS), pollen germinated (GER), pollen growing over the stigma surface (STA), pollen tubes located in upper half of style (UST), the lower half of style (LST), and in the locule (LOC).
[b]Number of (female × male) crossing combinations investigated.
[c]Time in hours after pollination.
[d]Number of pistils in which ovule micropyle penetration (fertilization) was observed.
[e]Total number of pistils observed by fluorescence microscopy.

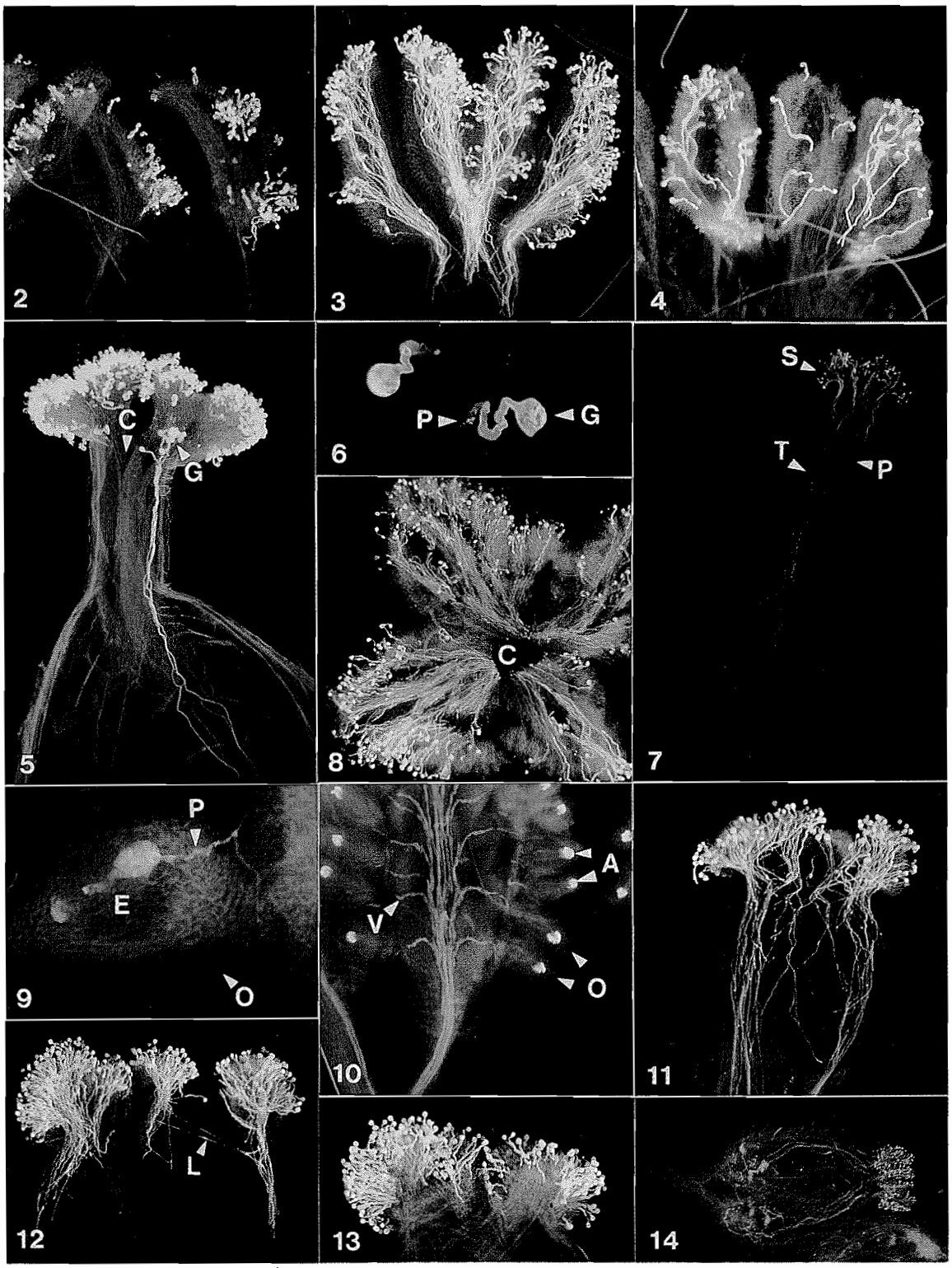
species' pollen, demonstrating that pollen–pistil incongruity was not only a function of species' crossability, but varied with the specific combining ability of parental genotypes within a species combination. In contrast, the pollen tubes of *S. amygdaloides* were highly compatible with *S. lucida* (Fig. 11), and although their growth rate was somewhat slower than conspecific pollen (Table 4), fertilization was observed in many crosses between these species. *Salix amygdaloides* belongs to the taxonomic section *Humboldtianae*, a section that is only represented in the western hemisphere (Dorn 1976), whereas *S. lucida* (section *Salicaster*) is closely related, morphologically, to the European species *S. pentandra*, from which it may have been derived. Pollen–pistil congruity relationships generally support the closer affinity of *S. amygdaloides* and *S. lucida* within subgenus *Salix* and the classification of the other species studied within subgenus *Vetrix*.

In *S. petiolaris*, pollination by *S. amygdaloides* and *S. lucida* resulted in the appearance of a large number of highly fluorescent spots in the stigma papillae, probably as a result of callose (1,3-β-glucan) deposition, and very poor pollen adhesion to the stigma. The high degree of incongruity between these species may be indicative of a stronger incongruity between the two subgenera. Poor pollen adhesion has been reported in intersectional crosses of *Populus* (Stettler *et al.* 1980; Gaget *et al.* 1984). The pollen tubes of *S. discolor* were unable to grow over the stigma of *S. petiolaris*, advancing very little beyond 12 h.a.p. In contrast, a very high degree of pollen–pistil congruity was observed in crosses where either *S. eriocephala* or *S. exigua* were used as pollen parents (Fig. 14). These crosses often led to fertilization.

In a genus of more than 500 species (Argus 1986), many of which occur in multispecies congeneric associations, reproductive relationships are of ecological and evolutionary interest.

Hogenboom (1973) viewed interspecific pollen–pistil incongruity as a result of the process of evolutionary divergence, and not as a response to selection for reproductive isolation, i.e., the Wallace effect (Grant 1971). However,

FIGS. 2–14. Fluorescent micrographs of interspecific pollen–pistil interactions in *Salix* spp. Fig. 2. Inhibition of pollen tube growth of *S. eriocephala* (clone 65) by the stigma of *S. discolor* (clone 14): stigmatic incongruity at 48 h after pollination (h.a.p.) ×30. Fig. 3. Stylar incongruity in *S. discolor* (clone 58) against pollen of *S. eriocephala* (clone 57) at 48 h.a.p. ×30. Fig. 4. Stigmatic incongruity in *S. discolor* (58) × *S. exigua* (63) at 24 h.a.p. ×30. Fig. 5. Omnipresent stigmatic barrier in *S. eriocephala* against pollen of all foreign *Salix* spp. Note germination and pollen tube growth of pollen grains (G) lodged in the open style canal (C) at 24 h.a.p. ×30. Fig. 6. Swollen, coiled, and disoriented pollen tubes (P) of *S. discolor* on the stigma of *S. eriocephala*. ×115. Fig. 7. Flower pistil in a *S. lucida* × *S. lucida* cross showing pollen tubes (P) streaming over the stigma (S), through the style (T), and into the locules at 48 h.a.p. ×12. Fig. 8. A surface view of pollen tubes (*S. exigua*, clone 42) streaming over the stigmatic lobes into the opening of the style canal (C) at 24 h.a.p. ×30. Fig. 9. A fertilized embryo sac (E) and the remnant of a pollen tube (P) entering the ovule (O) micropyle in *S. lucida* × *S. lucida* at 24 h.a.p. ×115. Fig. 10. Arrangement of unfertilized ovules (O) in one of the two locules of *S. lucida* showing the vascular connections (V) and the antipodal cells (A) of the ovules ×30. Fig. 11. The fully compatible pollen of *S. amygdaloides* streaming through the style of *S. lucida* at 24 h.a.p. ×30. Fig. 12. Stylar incongruity in *S. lucida* × *S. exigua* at 48 h.a.p. (L = callose plugs). ×30. Fig. 13. Stigmatic incongruity in *S. lucida* × *S. eriocephala* at 48 h.a.p. ×30. Fig. 14. Full pollen–pistil congruity and fertilization in *S. petiolaris* (53) × *S. exigua* (42) at 48 h.a.p. ×30.



the existence of a genetic mechanism for the inhibition of foreign pollen on the stigma surface provides an obvious selective advantage against the deleterious effects of hybridization in *Salix* (Mosseler 1987). An attempt should be made to isolate the compound(s) that may be responsible for the strong stigmatic barrier observed in *S. eriocephala*. The presence of such compounds demonstrates that the flower stigma has an important adaptive function in minimizing the wasted reproductive effort that often results from interspecific hybridization.

Mosseler and Papadopol (1989) showed that the seasonal timing of flowering may be an important premating reproductive barrier between the early flowering willows (*S. bebbiana*, *S. discolor*, *S. eriocephala*, and *S. petiolaris*) and the later flowering species (*S. amygdaloides*, *S. exigua*, and *S. lucida*). However, within these two groups there was substantial overlap in the seasonal flowering period. Results presented here demonstrate that pollen–pistil incongruity can be an important internal (postmating) barrier to hybridization between species that are not seasonally isolated. Reduced pollen tube growth rate alone would restrict the competitive ability of foreign species' pollen under natural pollination conditions and thus restrict hybridization in most natural populations.

Salix exigua has been classified together with *S. amygdaloides* and *S. lucida* in subgenus *Salix* by Dorn (1976), yet the results of pollen–pistil congruity suggest a higher genetic affinity between *S. exigua* and species of subgenus *Vetrix* than for species of subgenus *Salix*. Pollination barriers between *S. exigua* (as the pistillate parent) and pollen parents of species from subgenus *Vetrix* were not investigated because these crosses often yielded viable progeny (Mosseler 1987). The phylogenetic relationships described by Dorn (1976) were generally reflected by the degree of pollen–pistil congruity demonstrated between the willows studied. However, natural selection for genetic mechanisms that restrict hybridization between closely related sympatric species may confound the correlation between pollen–pistil congruity and phylogenetic relationships.

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