Pollen tube growth in tristylos  *Pontederia cordata* (Pontederiaceae)

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Pollen tube growth rates in legitimate cross- and self-pollinations were compared by controlled hand pollination in two varieties of *Pontederia cordata* L. (Pontederiaceae). In each pollen—stigma combination pollen germinated readily on stigmas and pollen tubes penetrated stigmatic tissue. Pollen tube length in styles was positively correlated with pollen size. In most pollinations legitimate pollen tubes grew more rapidly in styles than self pollen tubes. This difference was statistically significant in most comparisons. Inhibition of incompatible pollen tubes was either in the style or ovary. It is suggested that the mechanisms of incompatibility may differ among the floral morphs and depend on the particular pollen size involved in pollinations. The observed differences in pollen tube growth of legitimate cross- and self-pollinations in *P. cordata* suggest that the species is primarily outbreeding.


Les taux de croissance des tubes polliniques dans les pollinisation croisées légitimes et les autopollinisations ont été comparés par le biais de pollinisation manuelles contrôlées dans deux variétés du *Pontederia cordata* L. (Pontederiaceae). Dans chaque combinaison pollen—stigmate, le pollen a germé facilement sur les stigmates et les tubes polliniques ont pénétré le tissu stigmatique. La longueur du tube pollinique dans les styles était en corrélation positive avec la taille du grain de pollen. Dans la plupart des pollinations, le développement des tubes polliniques légitimes dans les styles était plus rapide que celui des tubes des autopollinisations. Cette différence était statistiquement significative dans la plupart des comparaisons. L’inhibition des tubes polliniques incompatibles se fait soit dans le style, soit dans l’ovaire. Nous proposons que les mécanismes d’incomaptabilité peuvent varier parmi les formes florales et dépendent de la taille particulière du pollen impliqué dans les pollinations. Les différences observées dans la croissance des tubes polliniques des pollinisations croisées légitimes et des autopollinisations chez le *P. cordata* indiquent que cette espèce est essentiellement xénogame.

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In most heterostylos plants the floral polymorphisms that constitute the heterostylos syndrome are associated with a sporophytically determined physiological self-incompatibility system (Lewis 1949; Vuilleumier 1967; Ganders 1979). Cross-pollinations between anthers and stigmas of equivalent height yield abundant seeds, whereas all remaining pollinations result in little or no seed set. Of importance to an understanding of the mechanisms governing these complex incompatibility systems are studies of pollen tube growth and the sites of inhibition for incompatible pollinations. Observations of this type are of particular interest in taxa with trimorphic incompatibility because of their unique intraflower pollen differentiation. Flowers of tristylos plants possess two anther levels from which pollen of contrasting size, number, and incompatibility behaviour is produced (Darwin 1877; Dulberger 1970; Barrett 1977; Weller 1980).

As part of a study of tristylos reproductive systems in the Pontederiaceae we have investigated various aspects of the pollination biology and mating behaviour of *Pontederia* spp. (Barrett 1977; Price and Barrett 1982, 1984; Glover and Barrett 1983; Barrett et al. 1983; Barrett and Anderson 1985; Barrett and Glover 1985; Wolfe 1985). Controlled crossing programmes conducted on four taxa of *Pontederia* have demonstrated that the three floral morphs display different levels of self-incompatibility and that pollen from the two anther levels within a flower exhibit different compatibility behaviour in self-pollinations (Barrett and Anderson 1985). Pollen loads of naturally pollinated stigmas of *Pontederia* are usually composed of compatible and incompatible pollen (Glover and Barrett 1983; Price and Barrett 1984; Barrett and Glover 1885). Thus, the question arises as to whether matings involving incompatible pollen might occur under field conditions. Because the anthesis period of individual flowers in *Pontederia* spp. is 6–8 h, rates of pollen tube growth of compatible and incompatible pollen are likely to be of critical importance in determining the mating system of populations. To investigate this problem we examined pollen tube behaviour in *P. cordata* using clonal material of *var. cordata* and *lancifolia* collected from the extremities of the North American range of the species. Here we describe methods for visualizing pollen tubes in the styles of *P. cordata* and quantify pollen tube growth in compatible and incompatible (self) pollinations. We also discuss the significance of the results to the mating system of the species and to the question of the phylogenetic relationships between heteromorphic and homomorphic incompatibility systems.

Materials and methods

**Plant material**

Experimental work was undertaken on clones of *Pontederia cordata* grown from seeds collected in two natural populations (*P. cordata* L. var. *cordata*, Paugh Lake, Ont.; and *P. cordata* var. *lancifolia* (Moh.) Torrey, 35 km southwest of Miami, FL). Nine clones (three per floral morph) were used from each population. Each clone was 4 years old (*var. cordata*) and 2 years old (*var. lancifolia*) at the time of experimental studies. Clones were a random sample of those used in a controlled crossing programme to determine levels of seed set from self- and cross-pollinations (Barrett and Anderson 1985). Clones were grown in plastic pots submerged in water-filled tubs placed on a single glasshouse bench under uniform growth conditions.

**Pollinations**

To quantify the expression of self-incompatibility in the styles of *P. cordata*, the growth of pollen tubes was compared after legitimate cross-pollination (between anthers and stigmas of equivalent height) and self-pollination with pollen from alternate anther levels within a
flower. The pollination treatments were conducted under glasshouse conditions (average temperature 25°C) during the winter and spring of 1983. On each of the 18 clones a total of 45 flowers from the same inflorescence for the posttime interval. Total included three pollination treatments (cross and selfs), three time intervals of pollen tube growth (2, 4, and 8 h), and five replicate flowers per treatment. The time intervals were chosen based on preliminary observations that indicated that these periods were most suitable for distinguishing the pollination treatments. On a given day 15 flowers on an inflorescence received the three pollination treatments and all flowers were removed after the time interval and fixed in 1:8:1 formalin — 80% ethanol — glacial acetic acid (FAA). For each inflorescence this procedure was repeated for 3 consecutive days with only the time period for pollen tube growth varying.

Observation of pollen tubes

Pollen tubes were visualized using a modification of the technique of Martin (1959). Pistils were fixed for 24 h or longer in FAA, rinsed in distilled water, softened and cleared for 24 h in a saturated solution of NaOH (8 M), rinsed again in tap water, and preserved in toluidine blue (0.05% in benzole buffer) for 1 to 2 min. Pistils were then rinsed in tap water and stained in aniline blue (0.01% in 0.01 M K,PO, for 4 h or more, before being mounted on a microscope slide in a drop of stain. Specimens were gently squashed to spread stylar tissue and enable pollen tubes to be viewed. Observations were made using an epifluorescent microscope (Reichert, Nr. 355 695) equipped with a HBO 50W/AC Super Pressure mercury lamp for UV illumination with excitor filter BG3 (maximum transmittance 365 nm) and barrier filter No. 3 (an orange filter). Aniline blue positive staining material (probably collors) occurs as plugs along the length of pollen tubes and enables them to be followed in the style.

Preliminary observations indicated that quantification of pollen germination on the stigmas and estimates of the total number of pollen tubes in styles were technically difficult because of the large numbers of pollen grains and pollen tubes involved. As a result, a single parameter, the distance travelled by the longest pollen tube, was recorded for each pollinated flower. Because P. cordata flowers possess a single ovule, it seems reasonable to assume that this measure has some relevance to the fertilization schedule. The distance travelled by the longest pollen tube was quantified by counting the number of fields of view through which the pollen tube passed and making this value relative to the length of the style.

The distance travelled by the longest pollen tube was compared among pollination treatments within each floral morph of both varieties. For the 2-h time interval a 3 × 2 contingency table was used with three pollination treatments and two sites of terminal pollen tube growth as contrasts. The sites of terminal pollen tube growth were reduced from four (see Table 1) to two because the frequencies of pistils in each of the four different categories were too low to conduct valid statistical tests. Pollination treatment and site where the longest pollen tube was observed were tested by chi-square to determine whether these factors were independent. A significant chi-square value implies that the pollination treatments result in different rates of pollen tube growth.

Results

General observations

Pollen germinated readily following both cross- and self-pollinations, indicating that strong inhibition of self-pollen does not occur on the stigma. The appearance of pollen tubes following the three pollination treatments was similar, with brightly fluorescing plugs laid down at unequal intervals along their entire length. Incompatible pollen tubes at the base of the style were often enlarged and curled. They frequently exhibited a loss in directionality of growth. Nevertheless, despite these effects, incompatible pollen tubes were frequently observed entering the ovary in certain incompatible pollen—stigma combinations (see below). These observations suggest that some form of ovarian inhibition occurs in Pontederia cordata. Pollen tubes penetrating the micropyle were, unfortunately, observed in very few pistils. The rarity of this observation is due to the squashing technique used to spread stylar tissue. The technique distorts ovarian tissue and usually prevents an unobstructed view of the micropyle. Maximum pollen tube length was observed to be dependent upon pollen grain size and style length. The greatest distance travelled by pollen tubes from small-sized pollen rarely exceeded 4 to 7 mm. Pollen from mid-level anthers generally produced pollen tubes with a maximum length of 7 to 9 mm and pollen from long-level anthers produced pollen tubes that grew to the base of the 14-mm style of the long-styled morph.

Analysis of pollen tube growth

Pontederia cordata var. cordata

Within each of the three floral morphs the three pollination treatments resulted in different pollen tube behaviour (Fig. 1). All cross-pollinated pistils of each morph had pollen tubes at the base of their styles after 2 h. This also occurred in short-styled pistils that had been self-pollinated with pollen from mid-level anthers. In the following self-pollinations (L × m/L, M × 1/M, S × 1/S) pollen tubes were observed at the base of the style after 4 h. Pistils of the L and M morph sampled 8 h after pollination with small-sized pollen rarely had pollen tubes at the base of their styles. For each time interval compatible pollen tubes generally had descended the styles more rapidly than in either of the two self-pollination treatments.

In the L and M morphs, the behaviour of pollen tubes from compatible and self-pollinations in pistils sampled after 2 h was significantly different (Table 1). Considerably more of the compatible pollinations resulted in pollen tubes at the base of the style. However, in the S morph the three treatments were not significantly different. A shorter time interval (e.g., 1 h) between pollination and fixation may be required to distinguish between treatments in this morph if a difference in pollen tube behaviour occurs.

Pontederia cordata var. lancifolia

The behaviour of pollen tubes in P. cordata var. lancifolia was similar to that observed in P. cordata var. cordata following the three pollination treatments (Fig. 2). The majority of pistils pollinated with compatible pollen had pollen tubes at the base of their styles when sampled after 2 h. Some differences were, however, evident in self-pollination treatments. Fewer pistils of the L morph had self pollen tubes at the base of their styles. When sampled 8 h after pollination, only 33% of the pistils pollinated with mid-sized pollen had pollen tubes that had grown down the entire length of the long style. Most pollen tubes of small-sized pollen were no further than half the length of the long style. Self-pollen from the two anther levels of the M morph also exhibited different pollen tube behaviour. All pistils sampled 8 h after pollination with large-sized pollen had pollen tubes at their bases. However, for the same sampling interval none of the pistils pollinated with small-sized pollen had pollen tubes further than three-quarters of the style length. In each of the floral morphs there is a statistically significant difference between pollination treatments sampled after 2 h (Table 2).

Discussion

The problem of the phylogenetic relationships between heteromorphic and homomorphic systems of sporophytic incompatibility has been the subject of considerable debate.
Crowe (1964), Muenchow (1981, 1982), and Wyatt (1983) have all argued that heteromorphic systems have arisen by loss of alleles from multiallelic homomorphic systems whereas Vuilleumier (1967), Ganders (1979), and Charlesworth (1982) postulate an independent origin from self-compatible ancestors. Most authors agree that heteromorphic incompatibility, which occurs in 24 unrelated families, is of polyphylectic origin.

One source of evidence to evaluate the evolutionary relationships between homomorphic and heteromorphic incompatibility comes from studies of the general properties of incompatibility reactions in the two systems. In particular, examination of pollen tube growth and sites of inhibition are important since similarities in behaviour could result from a common mechanism and provide evidence for ancestral relationships. In most cases families with homomorphic sporophytic incompatibility have trinucleate pollen and stigmatic inhibition of incompatible pollen. Studies of several unrelated genera exhibiting this system have demonstrated that pollen wall proteins, deposited by the tapetum, elicit the incompatibility reaction on the stigmatic surface soon after pollination occurs (e.g., Dickinson and Lewis 1973; Heslop Harrison et al. 1974; Howlett et al. 1973). In contrast, heteromorphic species may possess binucleate or trinucleate pollen (see Brebbaker 1967) and several different sites of pollen tube inhibition have been documented involving the stigma, style, and ovary (Lewis 1943; Esser 1953; Dulberger 1964, 1970; Shivanna et al. 1981; Ghosh and Shivanna 1982; Richards and Ibrahim 1982; Stevens and Murray 1982; Schou and Philipp 1983; Bawa and Beach 1983; Glover and Barrett 1983). These observations in conjunction with data on the taxonomic distribution of the two types of incompatibility (see Charlesworth 1982, 1985) cast doubt on the suggestion that heteromorphic systems are simply derived from homomorphic sporophytic systems by loss of alleles.

One of the most striking features of studies of pollen tube behaviour in heteromorphic taxa is the observation that the site of inhibition of incompatible pollen tubes often differs between the floral morphs (see references above). Inhibition of incompatible pollinations is often stigmatic in the short-styled morph and styrar in the long-styled morph (e.g., Bawa and Beach 1983). In our studies we could not detect differences in pollen germination between compatible and incompatible pollinations. Pollen tubes of both types of pollination readily penetrated stigmatic tissue. However, following penetration of the stigmatic tissue a series of inhibition sites were observed, depending on the particular pollen size — style length combination that was involved. Incompatible pollinations of the L and M morphs with small pollen (from short-level anthers) terminated growth in the styles after 8 h. This was confirmed by
TABLE 1. Contingency tables of the pattern of pollen tube growth in pistils sampled 2 h after pollination in *Pontederia cordata* var. *cordata*.

<table>
<thead>
<tr>
<th>Site where longest pollen tube was observed</th>
<th>Pollination treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long-styled morph</td>
<td>Cross L×l/M or S</td>
</tr>
<tr>
<td>Base</td>
<td>15</td>
</tr>
<tr>
<td>Style</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$x^2 = 31.49$, df = 2, $p &lt; 0.01$</td>
</tr>
<tr>
<td>Mid-styled morph</td>
<td>Cross M×m/L or S</td>
</tr>
<tr>
<td>Base</td>
<td>15</td>
</tr>
<tr>
<td>Style</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$x^2 = 35.66$, df = 2, $p &lt; 0.01$</td>
</tr>
<tr>
<td>Short-styled morph</td>
<td>Cross S×s/L or M</td>
</tr>
<tr>
<td>Base</td>
<td>15</td>
</tr>
<tr>
<td>Style</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$x^2 = 4.89$, df = 2, $p &gt; 0.05$</td>
</tr>
</tbody>
</table>

*In each pistil, the distance of the longest pollen tube was recorded.

*Base represents those pistils with a pollen tube at the base of the style. Style represents those pistils where the longest pollen tube terminated growth within the style.

TABLE 2. Contingency tables of the pattern of pollen tube growth in pistils sampled 2 h after pollination in *Pontederia cordata* var. *lancifolia*.

<table>
<thead>
<tr>
<th>Site where longest pollen tube was observed</th>
<th>Pollination treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long-styled morph</td>
<td>Cross L×l/M or S</td>
</tr>
<tr>
<td>Base</td>
<td>7</td>
</tr>
<tr>
<td>Style</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>$x^2 = 16.90$, df = 2, $p &lt; 0.01$</td>
</tr>
<tr>
<td>Mid-styled morph</td>
<td>Cross M×m/L or S</td>
</tr>
<tr>
<td>Base</td>
<td>13</td>
</tr>
<tr>
<td>Style</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>$x^2 = 23.22$, df = 2, $p &lt; 0.01$</td>
</tr>
<tr>
<td>Short-styled morph</td>
<td>Cross S×s/L or M</td>
</tr>
<tr>
<td>Base</td>
<td>12</td>
</tr>
<tr>
<td>Style</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>$x^2 = 6.03$, df = 2, $p &lt; 0.05$</td>
</tr>
</tbody>
</table>

*In each pistil, the distance of the longest pollen tube was recorded.

*Base represents those pistils with a pollen tube at the base of the style. Style represents those pistils where the longest pollen tube terminated growth within the style.

Observations made over longer time intervals. In contrast, incompatible pollinations with mid- and large-sized pollen frequently resulted in pollen tubes at the base of the style, particularly in the S morph. A similar pattern has also been observed in the related species *Pontederia sagittata* Presl (Glover and Barrett 1983). These observations indicate that there is an overall relationship between pollen size and pollen tube length in incompatible pollinations, suggesting that storage reserves may play a role in the incompatibility mechanism of some pollen–style combinations (e.g., L × s/L, M × s/M). Esser (1953) also documented a similar relationship in tristyloous *Lythrum salicaria* between pollen size and pollen tube length in self-pollinations.

If the distance attained by small pollen grains in styles of the L and M morphs is directly associated with storage products, the incompatibility mechanism may be largely passive in
nature. However, behaviour of the remaining incompatible pollinations with mid- and large-sized pollen suggests alternative mechanisms of inhibition since many pollen tubes rapidly descend the style and enter the ovary. While some incompatible pollinations result in moderate seed set (e.g., $M \times 1/M$), most do not (Ornduff 1966; Barrett and Anderson 1985). In the latter cases, it appears that some form of ovarian inhibition of self-pollinations is taking place. Further studies are required to determine the timing and cellular events leading to inhibition in ovaries and whether this behaviour is apparent in intermorph incompatible crosses. Of particular importance will be the determination of whether pre- or post-zygotic mechanisms are responsible for low seed set on selfing and if inbreeding depression is important (Seavey and Bawa 1986; Barrett 1986). Presumably, if ovarian inhibition is manifested in intermorph incompatible crosses, inbreeding depression can be ruled out.

In most comparisons made in our study, compatible pollen tubes grew more rapidly in styles than incompatible pollen tubes. Additional observations by us indicate that with shorter time intervals these differences are accentuated in some pollen–stigma combinations. Under field conditions, it is likely that the majority of matings would result from compatible (legitimate) pollinations, although under conditions of low flowering density or in large clones some selfing is likely to occur, owing to self-pollinations and geitonogamous pollinations (Price and Barrett 1984). Under these conditions the M morph would be most likely to self because of its moderate level of self-compatibility in self-pollinations with large-sized pollen. The consequences of selfing in the M morph to population structure and the maintenance of tristyly have been discussed by Charlesworth (1979) and Barrett et al. (1983).

The rates of pollen tube growth observed in P. cordata are rapid, with compatible pollen tubes entering the ovary between 1 and 2 h after pollination. Quantitative studies of the pollination process under field conditions in an Ontario population indicate that virtually all flowers are pollinated between 2 and 3 h after the beginning of anthesis and pollen tubes can be detected at the base of the style soon after (Wolfe 1985). Because P. cordata flowers are uniovulate and pollen loads average several hundred pollen grains it is possible that considerable gametophytic competition may occur (Barrett and Wolfe 1986). To what extent style length variation modulates the intensity of gametophytic selection in heteromorphic taxa is not known.

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