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FLOWER BIOLOGY OF SIX CULTIVARS OF THE BROMELIACEAE II. POLLINATION AND FERTILIZATION

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ABSTRACT. In describing the pathway followed by the pollen tube after compatible pollination, the authors found that pollen grains germinated on the papillate stigma and grew down in the style across stylar canal cells. They observed in the style of *Vriesea splendens,* a conversion from a hollow to a solid area at the lower end of the style. The pollen tube pathway of *V.* × *vimimalis-rex* × *carinata* and *V. splendens* differed from the universal pathway via the placenta described in literature and found by the authors for *Aechmea fasciata* and *Guzmania lingulata.* Fertilization occurred porogamously in all studied species. Pollen tubes of *A. fasciata* grew at 2 mm/hour, with those of the other cultivars at 1.1 mm/hour. The time between pollination and start of pollen tube growth was 5–6 hours for *Vriesea. Aechmea fasciata* was inter-compatible and, in part, self-compatible. The self-incompatibility index of *A. fasciata* decreased from 28% to 4% toward the end of the flowering period. Homomorphic gametophytic self-incompatibility is proposed for *A. chantinii* and for *Tillandsia cyanea.*

Key words: Bromeliaceae, incompatibility, fertilization, pollen tube growth, pollination

INTRODUCTION

Hybridization is the major source of phenotypic variation among ornamentals (Van Tuyl & De Jeu 1997). Fertilization barriers such as incompatibility and incongruity (cross-incompatibility), however, often prevent hybridization. Overcoming these barriers requires an understanding and illustration of the compatible pollination and fertilization process. The progamic phase includes the events occurring from pollen landing on the stigma to pollen discharge to the synergid (Shivanna 1982, Lord 2000). Pollen tube growth in the style is extremely fast, representing one of the most rapidly growing cells (Bedinger 1992, Sari-Gorla & Frova 1997). Pollen tubes extend by a tip-growth process. At regular intervals during tube elongation, callose plugs form, separating the living and growing part of the tube from the rest of the pollen tube (Cresti et al. 1992, Cheung 1996). A deceleration of pollen tube growth has been recorded when pollen tubes enter the ovary region (Herrero 1992), where they enter the ovule; and double fertilization occurs.

Following an incompatible pollination, the pistil will prevent fertilization by inhibiting either pollen germination, entry into the stigma, or tube arrest in the style or ovary (Shivanna 1982). Incompatibility can be defined as a genetic mechanism for preventing union of fertile gametes, in contrast to sterility, in which one or both gametes are not viable (Knox et al. 1986). Self-incompatibility (SI) prevents problems such as inbreeding depression and reduced gene pool variation (Franklin-Tong & Franklin 2000). Because self-incompatibility is regulated by S-genes, plant scientists have tended to think of SI as a qualitative trait of the breeding system—if a species has SI it is an obligate outcrosser. In reality, however, SI is often a quantitative trait (Stephenson et al. 2000). In both sporophytic and gametophytic SI, self-incompatibility occurs in degrees (Knox et al. 1986). Incompatibility takes place in all three bromeliad subfamilies, and self-compatible and self-incompatible species can belong to the same genus (Benzing 1980). *Vriesea* species including *V. splendens* were found to be capable of inbreeding, and many *Guzmania* spp. are self-pollinated (Smith & Downs 1974). The SI system of *Ananas comosus* is homomorphic gametophytic; whereas *A. ananassoides* and *A. bracteatus* are self-compatible (Brewbaker & Gorrez 1967).

Little is known about the compatible progamic phase and incompatibility level of Bromeliaceae. The aim of this study was to illustrate the progamic phase after compatible pollination. Special interest was given to pollen tube growth rate in the style and ovary. We discuss the incompatibility level of six cultivars belonging to four genera and two subfamilies. The results of this study may lead to improved breeding efficiency of Bromeliaceae (Vervaeke et al. 2002a, 2002b).

MATERIAL AND METHODS

Cultivars *Aechmea fasciata* and *A. chantinii* (Bromelioideae), *Vriesea* × *vimimalis-rex* ×

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carinata, V. splendens, Guzmania lingulata var. *minor,* and *Tillandsia cyanea* (Tillandsioideae) were studied. *V.* × *vimimalis-rex* × *carinata* and *G. lingulata* var *minor* are abbreviated as respectively *V. vr* × *carinata* and *G. lingulata.* Flowers were fixed in ethanol (70%). Dehydrated flower parts were critical point dried to study surface morphology of pollen grains growing in stigma, style, and ovary. Flower parts were examined using a JEOL JSM-5800 LV scanning electron microscope at 15 kV at the Nationale Plantentuin in Meise, Belgium.

To determine pollen tube growth rate, flowers of *Aechmea fasciata* were inter-pollinated, and those of *Vriesea vr* × *carinata, V. splendens,* and *Guzmania lingulata* were self-pollinated. At least 30 flowers were hand-pollinated in vivo at anthesis. Flowers were fixed in ethanol (70%) after different time intervals. To study the self-incompatibility level of *A. fasciata,* flowers at anthesis were self-pollinated (pollen derived from the same flower) and inter-pollinated (pollen derived from a flower of another plant within the same cultivar). These pollinations occurred at the beginning (1st week when flowers started to bloom on the inflorescence), the middle (2nd and 3rd weeks), and the end (4th week) of the flowering period. Flowers were fixed in ethanol (70%) 3 days after pollination. Ovules and pollen tubes were visualized by fluorescence microscopy. Pistils were incubated for 30 min in 4N NaOH (maceration) and stained with aniline blue (0.05% AB in 0.06 M K_2HPO_4-K_3PO_4, pH 11) for at least 16 hours. Pollen tubes were observed by means of UV fluorescence microscope (OLYMPUS BX 40). The tip of the longest pollen tube was marked with ink on the cover glass, and the distance from the beginning (upper part) of the style was estimated as approximate pollen tube length (Kuboyama et al. 1994). Ovules were considered fertilized when a pollen tube entered the micropyle.

Results and Discussion

The Progamic Phase

After compatible pollination, the pollen of *Aechmea fasciata* and *Vriesea splendens* hydrated and germinated. Pollen tubes emerged through one of the apertures of *A. fasciata* (Figure 1A) or the sulcus of *V. splendens* (Figure 1B). In a hollow style, as in Bromeliaceae and many other monocotyledons, the route for pollen tube growth in the style is through the stylar canal. This canal extends downward from the stigma (Figure 1C) (Shivanna et al. 1997). Within the stylar canal, pollen tubes occupy a superficial position with respect to cells bordering the canal, the so-called canal cells (Figure 1D); tubes are embedded in exudate (the extracellular matrix or ECM) produced by these cells (Labarca & Loewus 1973, Cheung 1996). The ECM is enriched in secreted materials, including sugars, amino acids, and fatty acids (Cheung 1995). During pollen tube growth, the ECM provides nutrient incentives and directional signals (Cheung 1996, Cheung et al. 2000). In a transverse section, the canal cells are circular and separated from one another (Shivanna 1982), as in *V. splendens* (Figure 1D).

The style of *Vriesea splendens* is solid at its proximal end (Figure 2A), forcing pollen tubes to grow through solid transmitting tissue to enter the ovary. At this location in the style, pollen tubes grew intercellular, i.e., between the cells of the transmitting tissue (Van Went & Willemse 1984, Richards 1997). Functionally, the difference between solid and hollow styles is minor. The intercellular substance in a solid style, a secretion product of the transmitting tissue, may be compared with the ECM of hollow styles (Sassen 1974, Raghavan 1997). The conversion from a hollow style to a closed style, as in *V. splendens* is not widespread. The function of this changeover tissue remains unclear. In different crossing experiments, however, pollen tubes were found to be inhibited at this point in the *V. splendens* style (I. Vervaeke unpubl. data). In some species, the style tissue breaks down lysogenously to different degrees, with the style showing a transition from solid to hollow type, as in *Vigna unguiculata* (Ghosh & Shivanna 1982); or it becomes completely hollow as in *Crotalaria retusa* (Malti & Shivanna 1984).

Before pollen tubes of *Vriesea splendens* entered the ovary (Figure 2B), they travelled along the ovary wall and surfaced close to the upper end of the funiculus. Then they grew over the ovules into the micropyle (porogamy) via the placenta (Figure 2C, D). More than one pollen tube entered the micropyle of some of the ovules of *V. splendens* (Figure 2C). In species with crassinucellate ovules, as in Bromeliaceae, several pollen tubes have been found to reach the nucellus, but only one tube penetrates it (Cresti et al. 1992). The end of pollen discharge is signalled by the formation of a callose plug over the pore preventing more tubes to penetrate the embryo sac (Raghavan 1997). In *V. splendens,* obturators were observed (Figure 2B, C). Very little is known about these secretory structures. By forming a protuberance between the ovule and the flat placenta, the obturator bridges the gap between the placenta and the micropyle, thereby facilitating pollen tube growth (Herrero 1992). Calcium is necessary not only for pollen germination but also for pollen tube growth and development

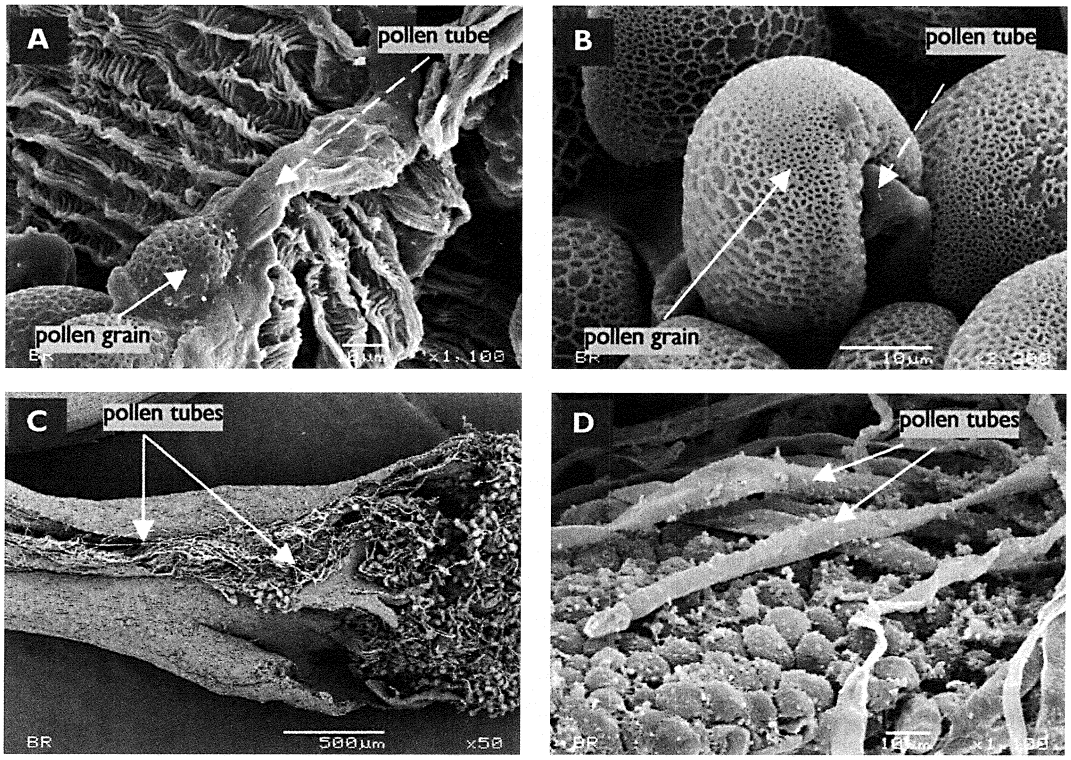

FIGURE 1. **A.** Pollen germination on *Aechmea fasiata* stigma, 2 days after pollination (×1100). **B.** Pollen germination on *Vriesea splendens* stigma, 3 days after pollination (×2300). **C.** Pollen germination on stigma and pollen tube growth in *V. splendens* style, 3 days after pollination (×50). **D.** Pollen tube growth on the canal cells of the hollow style of *V. splendens,* 3 days after pollination (×1100).

(Brewbaker & Kwack 1963, Bednarska 1991). Apical Ca^{2+} gradients and localized Ca^{2+} influx at the tip are accepted widely as general characteristics of growing pollen tubes (Cheung 1995, Franklin-Tong 1999a, 1999b). The presence of Ca-oxalate in the style of *V. splendens* (FIGURE 2E) and the ovary of *Aechmea fasciata* (FIGURE 2F) may be associated with this calcium requisite for pollen tube growth.

The pollen tube pathway for *Vriesea vr* × *carinata, V. splendens, Aechmea fasciata,* and *Guzmania lingulata* after compatible pollination was visualized (FIGURE 3). Pollen tubes of *V. splendens* entered the ovary via the ovary wall and grew over the ovules (FIGURE 3A, B). At a certain point, pollen tubes grew between the ovules to the placenta and fertilized ovules via the micropyle (porogamy). A similar pollen tube pathway in the ovary was found for *V. vr* × *carinata.* In most described multiovulate species, the pollen tube after reaching the ovary grows along the surface of the placenta towards the ovules (Knox et al. 1986, Webb & Williams 1988, Cresti et al. 1992, Sage & Williams 1995, Cheung 1996, Raghavan 1997, Shivanna et al. 1997, Herrero 2000). Examples include the pollen tubes of *A. fasciata* (FIGURE 3C) and *G. lingulata* (FIGURE 3D). Only Shuraki and Sedgley (1997) and Martin and Herscovitsch (1989) report on the ovary wall as a route for pollen tube growth in *Pistacia vera* and *Grevillea banksii,* respectively. In *P. vera* the ovule is entered via the chalaza (chalazogamy) and not by the micropyle as in *Vriesea.*

In our study, cells similar to the canal cells were observed on the inner ovary wall and may provide the same function, i.e., nutrition and guiding. Pollen tubes also grew over the ovules, but without any observable cells specialized in pollen tube growth. Pollen tubes may be attracted to the micropyle while growing over the ovules. Ovule micropyles are covered by an abundance of exudate enriched in sugar-containing substances that may contribute to pollen tube attraction (Franssen-Verheijen & Willemse 1993). A high concentration of Ca is associated with the synergid cells, where the rupture of one of these cells before pollen tube entrance creates a high local concentration of Ca around the micropyle (Chaubal & Reger 1990, 1992). These concentrations

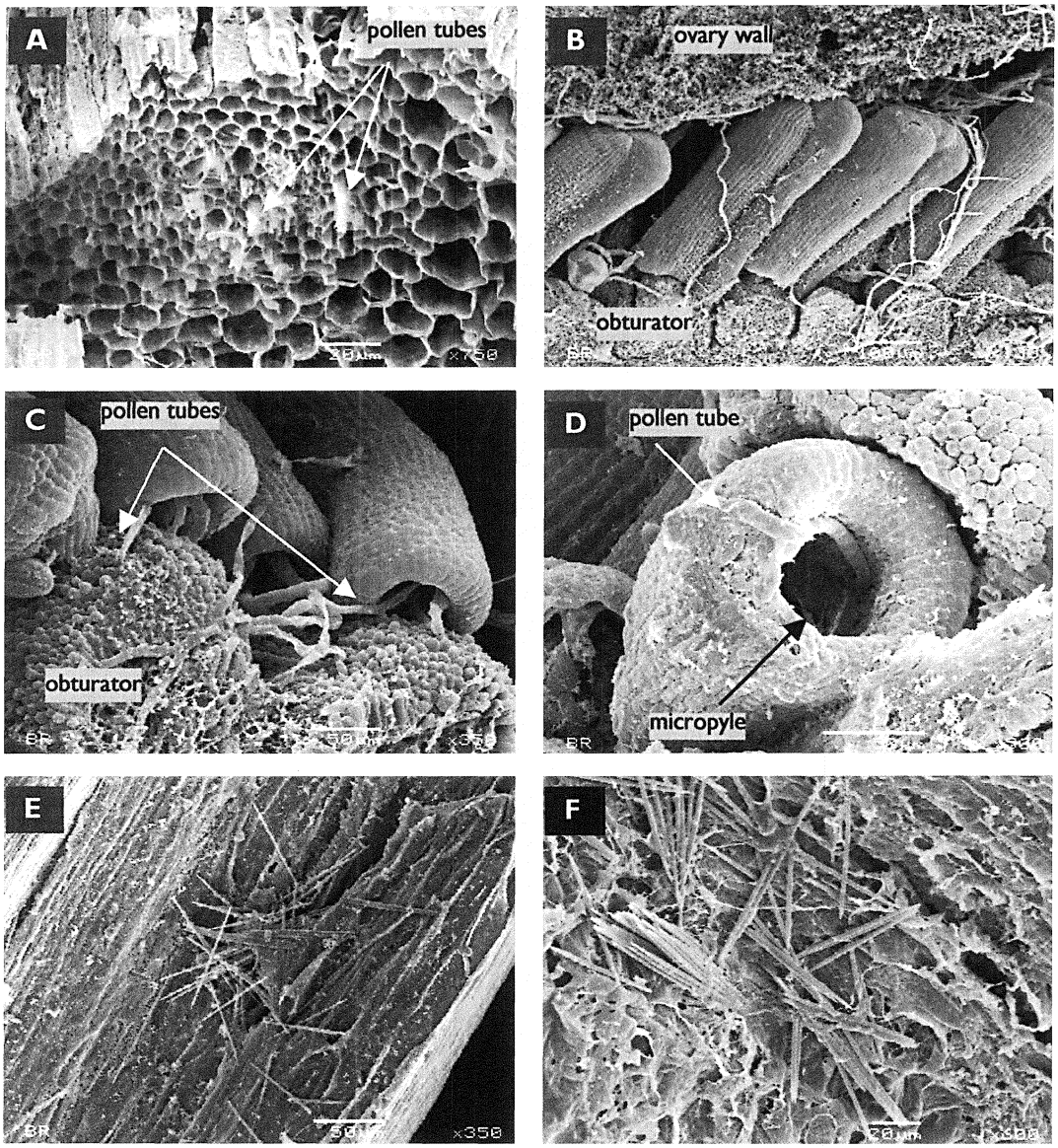

FIGURE 2. **A.** Pollen tube growth at the end of the *Vriesea splendens* style (transversal section), 3 days after pollination (×750). **B.** Pollen tubes in the ovary on *V. splendens* ovules (×130), 3 days after pollination. **C.** Pollen tube penetrates the ovules of *V. splendens* (×350), 3 days after pollination. **D.** Penetration of the micropyle by a pollen tube of *V. splendens* (×500), 7 days after pollination. **E.** Ca-oxalate crystals in style of *V. splendens* (×350). **F.** Ca-oxalate crystals in ovary of *Aechmea fasciata* (×600).

alone, however, do not explain how a directional signal could be provided (Russell 1996).

Pollen Tube Growth Rate in the Style

Pollen tube growth rate was determined after compatible in vivo inter-pollination of *Aechmea fasciata* and self-pollination of *Vriesea vr* × *carinata, V. splendens,* and *Guzmania lingulata* (FIGURE 4). After germination, pollen tubes started their journey in the stylar canal towards the ovary. Linear pollen tube growth rates in the styles were detected. *Aechmea fasciata* and *G. lingulata* pollen tubes started to elongate immediately after pollination. *Vriesea* pollen tubes did not extend until approximately 5–6 hours after pollination. These data were extrapolated by linear regression. For all studied cultivars, a limited number of pollen tubes

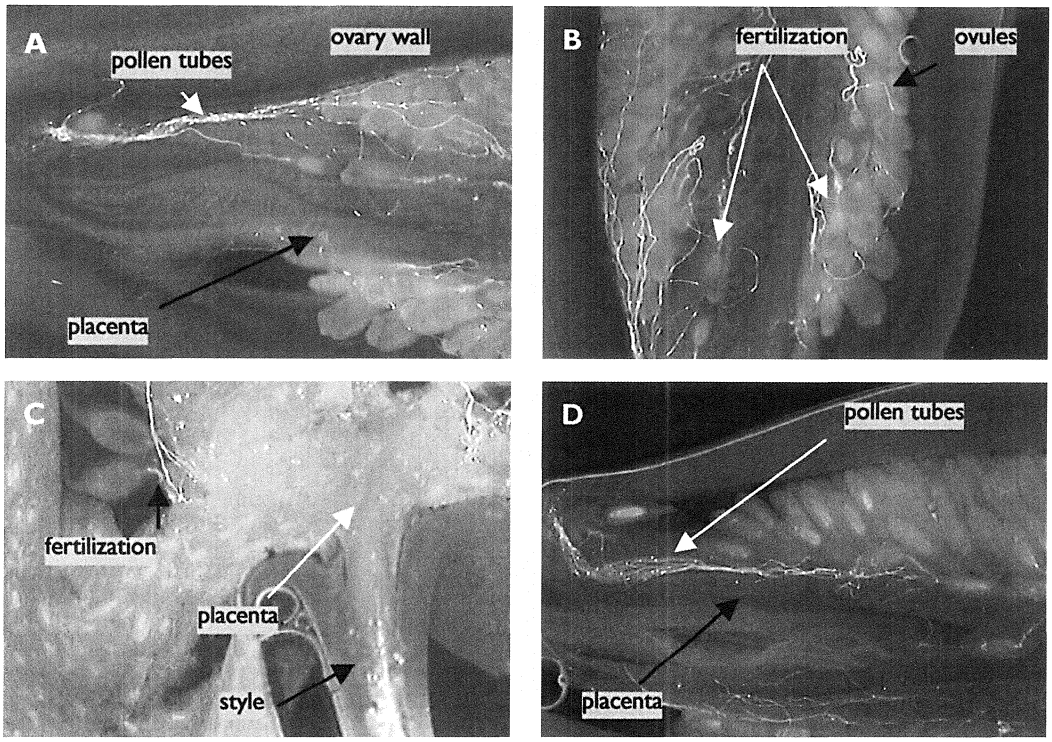

FIGURE 3. **A.** Pollen tube growth in the ovary of *Vriesea splendens* (4×10). **B.** Pollen tube growth and fertilization of *V. splendens* ovules (10×10). **C.** Pollen tube growth in the ovary of *Aechmea fasciata* (4×10). **D.** Pollen tube growth in the ovary of *Guzmania lingulata* (4×10).

(1–10) had reached the indicated length. When the style was observed closer to the stigma, the number of pollen tubes gradually increased; these pollen tubes may have a pioneer function and may activate the style tissue. Pollen tubes of *A. fasciata* grew at 2 mm/hour and those of the other cultivars at 1.1 mm/hour (FIGURE 4). Bromeliads generally show pollen tube growth in vitro, that is logarithmic with a maximum growth rate of approximately 200 μm/hour for *A. fasciata* (Parton et al. 2002). Pollen tubes cultured in vitro lack the biochemical, physiological, and physical environment of the pistil (de Graaf et al. 2001); therefore pollen tubes grown on artificial media generally do not grow as fast or as long as those grown in vivo (Sari-Gorla & Frova 1997). Estimates made by linear extrapolation suggest that the first pollen tubes reached the style end at 14 hours for *A. fasciata,* 46 hours for *V. vimimalis rex* × *carinata,* 48 hours for *V. splendens,* and 31 hours for *G. lingulata.* With time the number of pollen tubes at the stylar end increased (FIGURE 4).

Incompatibility Status

Aechmea fasciata was inter-compatible, but fertilization rates decreased after the first week of bloom (TABLE 1). Self-incompatibility was observed in *A. fasciata,* along with limited fertilization rates. The percentage of ovules fertilized decreased for successive flowers in the same inflorescence. Pollen tube inhibition was confined at approximately one-fourth of the style. The index of self-compatibility (ISC) is calculated as the ratio of seed obtained by controlled self-pollination to seed obtained by cross-pollination (Knox et al. 1986, Stephenson et al. 2000). The ISC decreased from 28% to 4% between the beginning and end of the flower period. The floral position within an inflorescence had no effect on the strength of SI in *Campanula rapunculoides* (Stephenson et al. 2000). Flower characteristics of *A. fasciata* such as petal length, pistil length, ovary width, and number of ovules decreased as the flowering period progressed. In a previous study, we found that in vitro pollen germination, however, did not decrease. Pollination of aging flowers is a method that may yield occasional self-seed in SI systems. This effect may represent breakdown with age of the specific molecular determinants of the self-incompatibility response. If ethylene stimu-

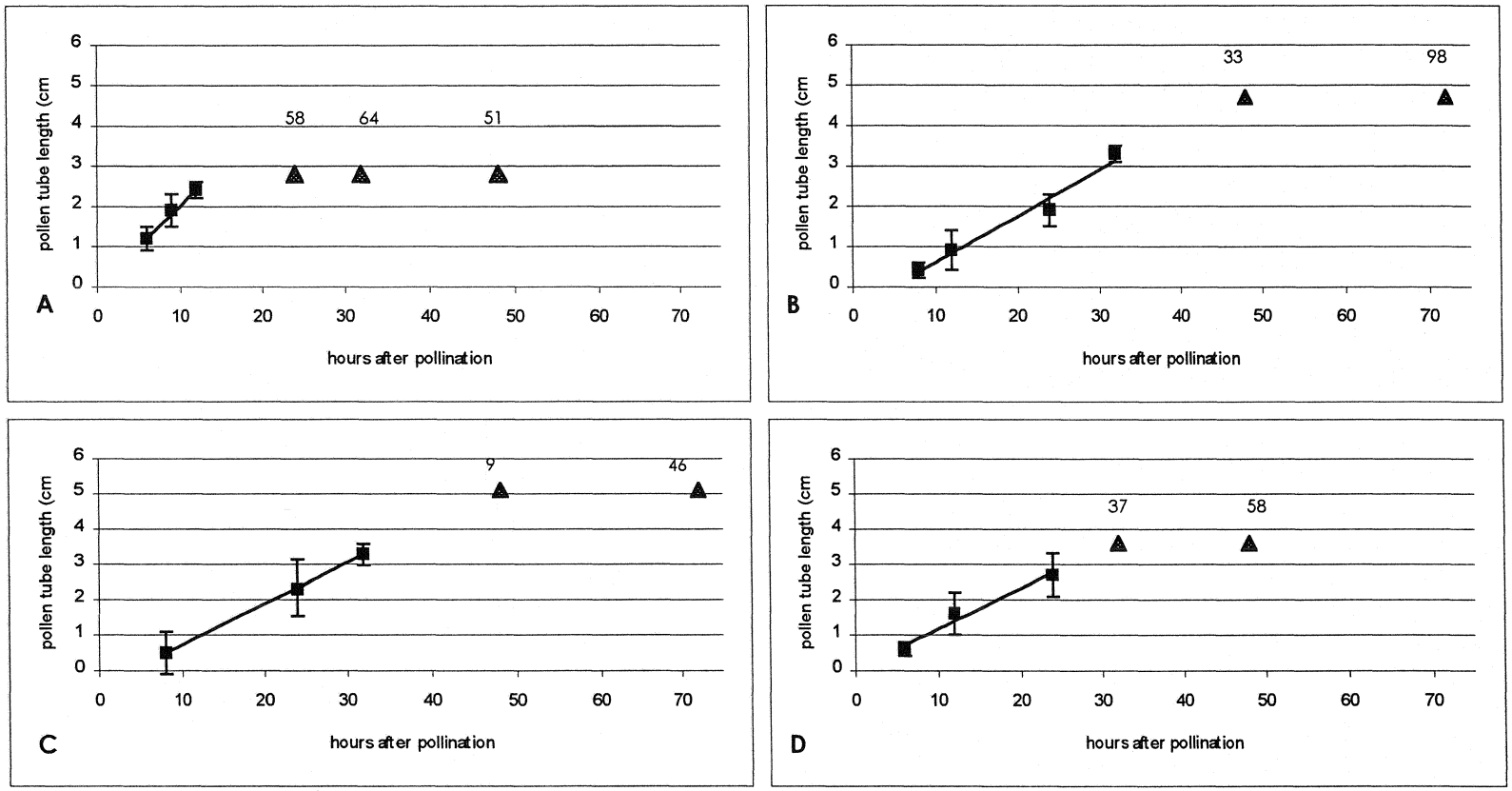

FIGURE 4. Pollen tube growth rate (cm/hour) in style (■) and number of pollen tubes at the style end (▲) in relation to different hours after pollination of **A.** *Aechmea fasciata.* **B.** *Vriesea* × *vimimalis- rex* × *carinata.* **C.** *Vriesea splendens.* **D.** *Guzmania lingulata* (arrows indicate style length).

TABLE 1. Percentage ovules penetrated by a pollen tube (Fertilization %) and fertilization index (FI %) of *Aechmea fasciata* after inter- and self-pollination in vivo (at anthesis) during beginning, middle, and end of flowering period of inflorescence (April–July 2001).

Pollination	Flower period	n	Fertilization (%)	FI (%)
Inter-	Beginning	36	32 ± 18[a]	92
	Middle	42	22 ± 18[b]	79
	End	29	25 ± 21[b]	76
Self-	Beginning	42	9 ± 17[c]	69
	Middle	53	3 ± 7[cd]	27
	End	28	1 ± 4[d]	24

Note: n = number of pollinations observed; ± = SE of means; same letters in column = no significant difference based on Duncan's multiple range test; and FI = % ovaries where one or more ovules were fertilized.

lates pollen tube growth, however, the effect might be the result of increased ethylene production associated with senescence (Williams & Webb 1987). Aging of the inflorescence and most likely increased ethylene production had a negative effect on the fertilization rate by both compatible and incompatible pollen.

Previous studies also found that *Aechmea chantinii* is self-incompatible (Vervaeke et al. 2001). *Tillandsia cyanea* was considered self-incompatible by Vervaeke et al. (2001), however, after in vivo inter-pollination, some pollen tube penetration was observed (Parton et al. 2001). The inhibition reaction in both cultivars occurred in the style, which is characteristic for gametophytic self-incompatibility (de Nettancourt 1977, Newbegin et al. 1993). The SI system of *Ananas comosus* was determined as homomorphic gametophytic SI (Brewbaker & Gorrez 1967). Distributions across Magnoliophyta suggest that neither homomorphic, nor heteromorphic, sporophytic SI are likely to occur in Bromeliaceae, although poorly understood late-acting SI systems cannot be ruled out (Benzing 2000). Sexual asynchronism, either as protandry or protogyny, did not occur in the studied cultivars, since both pollen and stigma were viable at anthesis (Parton et al. 2001). *Vriesea vr* × *carinata, V. splendens,* and *Guzmania lingulata* are self-compatible (Vervaeke et al. 2001). Because anthers and stigma are at the same level at anthesis, emasculation of all cultivars (except for *V. vr* × *carinata*) is required before anthesis to avoid self-pollination and self-fertilization.

CONCLUSIONS

During the progamic phase, pollen-pistil interactions are built up (Franklin-Tong 1999a, 1999b). These interactions assist with guidance of pollen tubes to the ovule micropyle (Vervaeke et al. 2002a, 2002b). The progamic phase of *Aechmea fasciata* and *Vriesea splendens* was visualized using Scanning Electron Microscopy (SEM). Several structures were observed, most likely in relation to pollen-pistil interactions: canal cells, Ca-oxalate crystals in style and ovary, and obturators on the placenta. Pollen tube growth rate after compatible pollination was determined. Pollen tube cultures in vitro grow slower and remain shorter, compared with the in vivo situation. The manifestation of self-incompatibility in *Aechmea fasciata* was variable. Aging of the inflorescence and most likely increased ethylene production had a negative effect on the fertilization after both self- and inter-pollination of *A. fasciata.* Because pollen tubes were inhibited in the style and taxonomic relation, homomorphic gametophytic self-incompatibility is proposed, as in *Ananas comosus* (Brewbaker & Gorrez 1967).

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