CONTROLLING PREFERTILIZATION BARRIERS BY IN VITRO POLLINATION AND FERTILIZATION OF BROMELIACEAE

I. Vervaeke, E. Parton, R. Deroose and M.P. De Proft
Laboratory of Plant Culture
Catholic University of Leuven
W. De Croylaan 42
B-3001 Leuven
Belgium
ine.vervaeke@agr.kuleuven.ac.be

Keywords: Bromeliaceae, cut style pollination, placental pollination, pollen-tube growth, *semi in vivo* pollination

Abstract

Prefertilization barriers between different Bromeliaceae are mostly confined within the style. To control these barriers cut style, placental and placental grafted style pollination were studied. After cut style pollination the fertilization rate increased when a longer style part was left on the ovary of *Vriesea* C. Possibly less factors that control pollen tube guidance to the ovules are present in the lower style part. After placental pollination pollen germinated on the ovules but only rarely penetration of the micropyle by a pollen tube occurred. No difference in fertilization rate between compatible and incongruent pollination was found. *Semi in vivo* pollination resulted in a higher pollen tube growth out of the isolated style in comparison to *semi in vitro* pollination. However for *Vriesea* C, even after *semi in vivo* pollination pollen tube growth was inhibited at the stylar end. Grafting a style containing actively growing pollen tubes to the ovules on the placenta (placental grafted style pollination) resulted in a fertilization percentage, which was comparable to this after a classical pollination on the stigma for *Aechmea* A. Pollen tube growth through the style seems to be necessary for guiding pollen tubes to the ovule micropyle.

1. Introduction

Bromeliads used as ornamentals, originate mostly from tropical and subtropical rainforests of Central and South America (Benzing, 1980). Understanding the nature of prefertilization barriers is a key element before manipulations can be designed to achieve hybridization. Prefertilization barriers between different Bromeliaceae are mostly confined within the style (Vervaeke *et al*., 2001a). A range of techniques, such as cut style pollination, grafted style pollination, placental pollination, use of mentor pollen, have been applied to overcome prefertilization barriers in ornamentals (Van Tuyl and De Jeu, 1997). The cut style technique comprises deposition of pollen on the stylar surface after removing a part of the style, including the stigma (Van Tuyl *et al*., 1991). Placental pollination involves removal of the stigma, style and ovary wall, pollination of the ovules on the placenta, and culturing of the ovule mass on a suitable medium (Rangaswamy and Shivanna, 1967).

The main significance of these alternative pollination techniques is that they provide means for bypassing the pollen-stigma or pollen-style rejection reaction seen in incompatible and incongruent crosses. The practice of grafting a style with growing pollen tubes to a placenta with ovules is called here 'placental grafted style pollination' (Vervaeke *et al*., 2001b).

2. Materials and methods

2.1. Plant material

Aechmea A (subfamily Bromelioideae), *Guzmania* A, *Tillandsia* A, *Vriesea* A and C (subfamily Tillandsioideae) were studied. Cultivar names are encoded (see Vervaeke *et al*., 2001a). Plants were cultivated in greenhouses and maintained at an average day and night temperature of 21 ± 0.5°C and 19.5 ± 0.5°C respectively, and at 65 ± 5% relative humidity. Normal nursery practice for pest control, watering, ventilation and fertilization were conducted. Flower induction was done by ethylene. *Aechmea* A originated from seed and was compatible after interpollination (pollen put on different plants all from the same species out of the same seedling population). *Guzmania* A and *Vriesea* B and C were self-compatible. *Tillandsia* A was considered self-incompatible by Vervaeke *et al.* (2001a), however after *in vivo* interpollination some pollen tube penetration was observed (Parton *et al*., 2001).

2.2. Microscopy

All ovaries and styles were fixed 2 days after pollination in ethanol (70%) for at least 24 hours. After washing out ethanol, pistils were 30 minutes incubated in 4N NaOH (maceration) and stained with aniline blue (0.05% AB in 0.06 M KH_2PO_4-K_3PO_4, pH 11) during minimum 16 hours in darkness. An ovule was considered fertilized when a pollen tube penetrated the micropyle. For every ovary the % ovules fertilized was determined. The fertilization index (FI) is the % ovaries where one or more ovules were penetrated by a pollen tube. The % ovules fertilized of FI is the % ovules fertilized when only the ovaries where fertilization occurred were considered. For statistical analysis evaluation Duncan's multiple range test was used.

2.3. Cut style pollination

Vriesea C flowers were pollinated *in vivo* at anthesis. The cut surface of the style was pollinated with fresh compatible pollen after removing 25%, 50%, 75% and 100% of the style. Removing 0% of the style refers to pollination on stigma. Pistils were fixed 5 days after pollination.

2.4. Placental pollination

Aseptic pollen was obtained by surface disinfections of a flower bud one day before anthesis. Anthers were aseptically removed and were kept in petri dishes under humid conditions for 24 hours. Flowers were isolated from the plant at anthesis. The flowers were disinfected by rinsing them in sterile water, 20 sec in ethanol (70%), rinsing with sterile water and immersing 15 minutes in NaOCl (4°). This was followed by three rinses in sterile distilled water. Petals, stamen and style were removed and an opening was made in the ovary to expose the ovules. Pollen grains were deposited directly onto the ovules. The ovary was then placed on modified Nitsch medium (Higashiyama *et al*., 1998) and fixed 2 days after pollination.

2.5. *Semi in vivo* and *semi in vitro* pollination

For *semi in vivo* pollination (Mulcahy and Mulcahy, 1985) flowers were pollinated *in vivo* at anthesis and after x hours the cut ends of the styles were immediately dipped in modified Nitsch medium (Higashiyama *et al*., 1998). *Semi in vitro* pollinated styles were detached from the ovary and were immediately put on medium after pollination on stigma *in vitro*. The pollen tubes protruded from the cut ends and grew as bundles. The average number and length of pollen tubes inside the medium was determined 2 days after

pollination.

2.6. Placental grafted style pollination

Flowers were isolated at anthesis from the plant. The style, petals and stamen were removed. The ovary was surface disinfected with ethanol (70%) and a slit was made in the ovary. Modified Nitsch medium (3 % w/v agar) was injected into the ovary holes and 2 *semi in vivo* pollinated styles (6 h – 1.5 cm) were placed in one opening so that the pollen tubes that grew out of the style would pass the ovules (Fig. 4A). The ovary was then placed on modified Nitsch medium. Two days after pollination pistils were fixed to determine the fertilization rate after staining with aniline blue.

3. Results

3.1. Cut style pollination

After compatible pollination at anthesis on the stigma of *Vriesea* C, on average 61% of the ovules were penetrated by a pollen tube (Table 1) and regular pollen tubes in the style were observed. Cut style pollination after removing 25% of the style resulted in a penetration of 37% of the ovules. Germination percentage on style and stigma did not differ, but after cut style pollination less pollen were deposited on the cut style surface in comparison to the stigma (Fig. 1A). Consequently less pollen tubes grew in the style. The penetration percentage of ovules by pollen tubes gradually decreased when a longer part of the style was removed. More abnormal pollen tubes were observed after removing 75% and 100% of the style, like strong callose deposition, swelling of the tube tip and curling. Also in the ovary pollen tubes were not guided to the ovule micropyle, pollen tubes sometimes grew in between the integuments instead of the micropyle (Fig. 1B).

3.2. Placental pollination

Ovules of ovaries at anthesis were pollinated. Pollen germination on the ovules occurred, however pollen tubes showed strong callose deposition and remained short (Fig. 2B). *Aechmea* A, *Vriesea* C and *Tillandsia* A were pollinated *in vitro* at anthesis with compatible and incongruent pollen (Table 2). Penetration of the ovules by pollen tubes occurred exceptional (Fig. 2A). The crossing of *Tillandsia* A x *Vriesea* C resulted in a fertilization index of 8%. Because approximate 400 ovules are present per ovary, the average fertilization % was 0. Both compatible and incongruent pollination resulted in failure of fertilization after placental pollination.

3.3. *Semi in vitro* and *semi in vivo* pollination

Pollination *in vivo* before placing isolated styles into *in vitro* medium (*semi in vivo* pollination) had a significant effect on number and length of pollen tubes that protruded from the cut stylar ends in comparison to *semi in vitro* pollination (Table 3, Fig. 3B). However also after *semi in vivo* pollination of *Vriesea* C pollen tube growth was inhibited at the style end (Fig. 3A).

3.4. Placental grafted style pollination

To supply the ovules on the placenta with active growing pollen tubes instead of pollen grains, styles were grafted on the placenta of *Aechmea* A (Fig. 4A). Pollen tube growth from the stylar end towards the ovules was not always successful (Table 4). Penetration of ovules by pollen tubes was comparable to that after compatible pollination on stigma (Fig. 4B). Placental grafted style pollination was repeated at different periods and with different plants. Variation can be explained by difference in quality of the pistil

(ageing of the inflorescence has a negative effect on pollen tube emergence from the style), this resulted in poorer pollen tube growth towards the ovules.

4. Discussion

After cut style pollination the percentage of ovules penetrated by a pollen tube was lower than after pollination on stigma. For *Vriesea* C fertilization rate decreased with a decreasing style length. This observation was also found after cut style pollination of *Aechmea* A (Vervaeke *et al*., 2001b) and *Lilium longiflorum* (Janson *et al*., 1993). This lower fertility could be due to the fact that less pollen can be deposited on the cut style surface and pollen tubes grow only through the shorter lower style part. *Semi in vitro* pollination on the cut surface of the lower style part showed lower amounts and lengths of pollen tubes that protruded from the cut style than after pollination of the upper half (without stigma). Probably less or other factors that control pollen tube growth are present in the lower in comparison to the upper style part (Vervaeke *et al*., 2001b).

Pollen germinated on the ovules after placental pollination but only occasionally penetration of the micropyle occurred. The low penetration percentage after placental pollination seems to have its basis in an inability of the pollen tube to react to signals from the micropyle (Janson, 1993). Pollen from many species germinates and grows in simple, chemically defined media. However, *in vitro* grown tubes neither attain the growth rates nor the distances reached by their *in vivo* counterparts (Cheung *et al*., 2000). In *Torenia fournieri* pollen tubes that had germinated *in vitro* elongated around ovules, but no pollen tubes reached the micropylar end of the naked embryo sacs (Higashiyama *et al*., 1998). Activation of the *Aechmea* A ovary by a compatible pollination of 6 hours or pollination 2 days after anthesis did not improve pollen tube penetration (Vervaeke *et al*., 2001b).

The *semi in vivo* method demonstrated the influence of the ovary on pollen tube growth in some species (Mulcahy and Mulcahy, 1985), whereas with others such influence seems to be absent (Kandasamy and Kristen, 1987). *Semi in vivo* pollination resulted in stronger pollen tube growth out of the style in comparison to *semi in vitro* pollination for the studied bromeliads. Compared with pollen tubes germinated *in vitro*, pollen tubes under *semi in vivo* conditions elongated in straight lines, at higher growth rates, and with smaller diameters (Higashiyama *et al*., 1998). The length of the pollen tube protruding from the cut style is enhanced by allowing initial pollen tube growth to occur before the ovary is removed as in *Petunia* (Mulcahy and Mulcahy, 1985). Possibly also senescence of the cut style plays a role in pollen tube behaviour (Vervaeke *et al*., 2001b). *Vriesea* C pollen tubes were inhibited at the stylar end even after *semi in vivo* pollination. This could be due to a wounding reaction at the cut stylar end. In maize, for instance, the wound at the cut end heals soon after the cut, so that the growing tube tip cannot emerge (Sari-Gorla and Frova, 1997).

Grafting a style with active growing pollen tubes resulted in a higher fertilization percentage in comparison to placental pollination. According to Higashiyama *et al*. (1998) germination of pollen on the stigma and passage of the tube through the style were essential for guidance to ovules *in vitro* of *Torenia fournieri*. When ovules are presented to pollen tubes emerging from *semi in vivo* pollinated styles, pollen tubes often show a preference for growing in direction of these ovules (Cheung *et al*., 2000).

It is clear that after cut style and placental pollination fewer interactions between pollen and pistil occur. Removing a possible barrier, i.e. stigma and style, creates another one. This new barrier is caused by the pollination technique itself. By pollination on the cut style or immediately on the ovules, compatible pollen tubes are not longer guided to the ovule micropyles, because no or less pollen-pistil interactions are built up (Vervaeke *et al*., 2001b). During pollen tube growth the enriched extracellular matrix (ECM) of the style provides nutrient incentives and directional signals (Cheung, 1996; Cheung *et al*., 2000).

Acknowledgements

This research is supported by the Ministry of Middle Class and Agriculture of Belgium, Committee of Research and Development (DG-6). The authors thank Reginald Deroose bvba for the financial support of this Research program.

References

Benzing D.H., 1980. The biology of the Bromeliads. Mad River Press Eureka, California.

Cheung A.Y., 1996. Pollen-pistil interaction during pollen-tube growth. Trends Plant Sci 1: 45-51.

Cheung A.Y., Wu H-M, Di Stillo V., Glaven R., Chen C., Wong E., Ogdahl J., and Estavillo A., 2000. Pollen-pistil interactions in *Nicotiana tabacum*. Ann Bot 85(A): 29-37.

Higashiyama T., Kuroiwa H., Kawano S., and Kuroiwa Y., 1998. Guidance *in vitro* of the pollen tube to the naked embryo sac of *Torenia fournieri*. Plant Cell 10: 2019-2031.

Janson J., 1993. Placental pollination in *Lilium longiflorum* Thunb. Plant Science 90: 105-115.

Janson J., Reinders M.C., Van Tuyl J.M., and Keijzer C.J., 1993. Pollen tube growth in *Lilium longiflorum* following different pollination techniques and flower manipulations. Acta Bot Neerl 42: 461-472.

Kandasamy M.K., and Kristen U., 1987. Pollen tube growth in the style of *Nicotiana sylvestris* is neither influenced by the ovary nor directed by a gradient. J Plant Physiol 131, 495-500.

Parton E., Vervaeke I., Deroose R., and De Proft M.P., 2001. Interspecific and intergeneric fertilization barriers in Bromeliaceae. Act. Hort. 552: 43-53.

Mulcahy G.B., and Mulcahy D.L., 1985. Ovarian influence on pollen tube growth, as indicated by the semivivo technique. Amer J Bot 72: 1078-1080.

Rangaswamy N.S., and Shivanna K.R., 1967. Induction of gametic compatibility and seed formation in axenic cultures of diploid self-incompatible species of *Petunia*. Nature 216: 937-939.

Sari-Gorla M, and Frova C, 1997. Pollen tube growth and selection. In: Shivanna, K.R. & Sawhney V.K. (eds.). Pollen biotechnology for crop production and improvement. Cambridge University Press, London, 333-351.

Van Tuyl J.M., Van Diën M.P., Van Creij M.G.M., Van Kleinwee T.C.M., Franken J., and Bino R.J., 1991. Application of *in vitro* pollination, ovary culture, ovule culture and embryo rescue for overcoming incongruity barriers in interspecific *Lilium* crosses. Plant Science 74: 115-116.

Van Tuyl J.M., and De Jeu M.J., 1997. Methods for overcoming interspecific crossing barriers. In: Shivanna, K.R. & Sawhney V.K. (eds.). Pollen biotechnology for crop production and improvement. Cambridge University Press, London, 273-292.

Vervaeke I., Parton E., Maene L., Deroose R., and De Proft M.P., 2001a. Prefertilization barriers between different Bromeliaceae. Euphytica, 118: 91-97.

Vervaeke I., Parton E., Maene L., Deroose R., and De Proft M.P., 2001b. Pollen tube growth and fertilization after different *in vitro* pollination techniques of *Aechmea fasciata*. Euphytica, in press.

Table 1. Germination on stigma or style (%), fertilization (%) after *in vivo* self-pollination on stigma (0% style removed) or cut style (25%, 50%, 75% or 100% style removed) of *Vriesea* C

Plant species	% style removed	% germination on stigma/style	% fertilization
Vriesea C	0%	36 ± 11 a	61 ± 28 a
	25%	37 ± 12 a	37 ± 25 b
	50%	43 ± 12 a	24 ± 13 c
	75%	41 ± 16 a	5 ± 4 d
	100%	41 ± 9 a	2 ± 3 d

a,b,c,d: Duncan's multiple range test (Pr = 0.05), mean ± SE (n = 30)

Table 2. Pollen germination on ovules (%), fertilization (%) and fertilization index (% ovaries where one or more ovules are penetrated by a pollen tube) after *in vitro* placental pollination at anthesis of *Aechmea* A (interpollination), *Tillandsia* C self pollination and cross-pollination with *Vriesea* A, *V.* C and *Guzmania* A and self pollination of *Vriesea* C

	% pollen germination on ovules	% fertilization	Fertilization index (%)
Aechmea A x *Aechmea* A	49 ± 21	0 ± 1	20
Tillandsia A x *Tillandsia* A	39 ± 17	0 ± 0	7
Tillandsia A x *Vriesea* A	43 ± 21	0 ± 0	0
Tillandsia A x *Vriesea* C	37 ± 18	0 ± 0	8
Tillandsia A x *Guzmania* A	43 ± 15	0 ± 0	0
Vriesea C x *Vriesea* C	41 ± 13	0 ± 0	0

Mean ± SE (n = 30)

Table 3. Average number and length (µm) of pollen tubes that grow beyond the cut stylar ends in modified Nitsch medium of *Aechmea* A, *Vriesea* A, *Vriesea* C and *Guzmania* A (2 days after pollination)

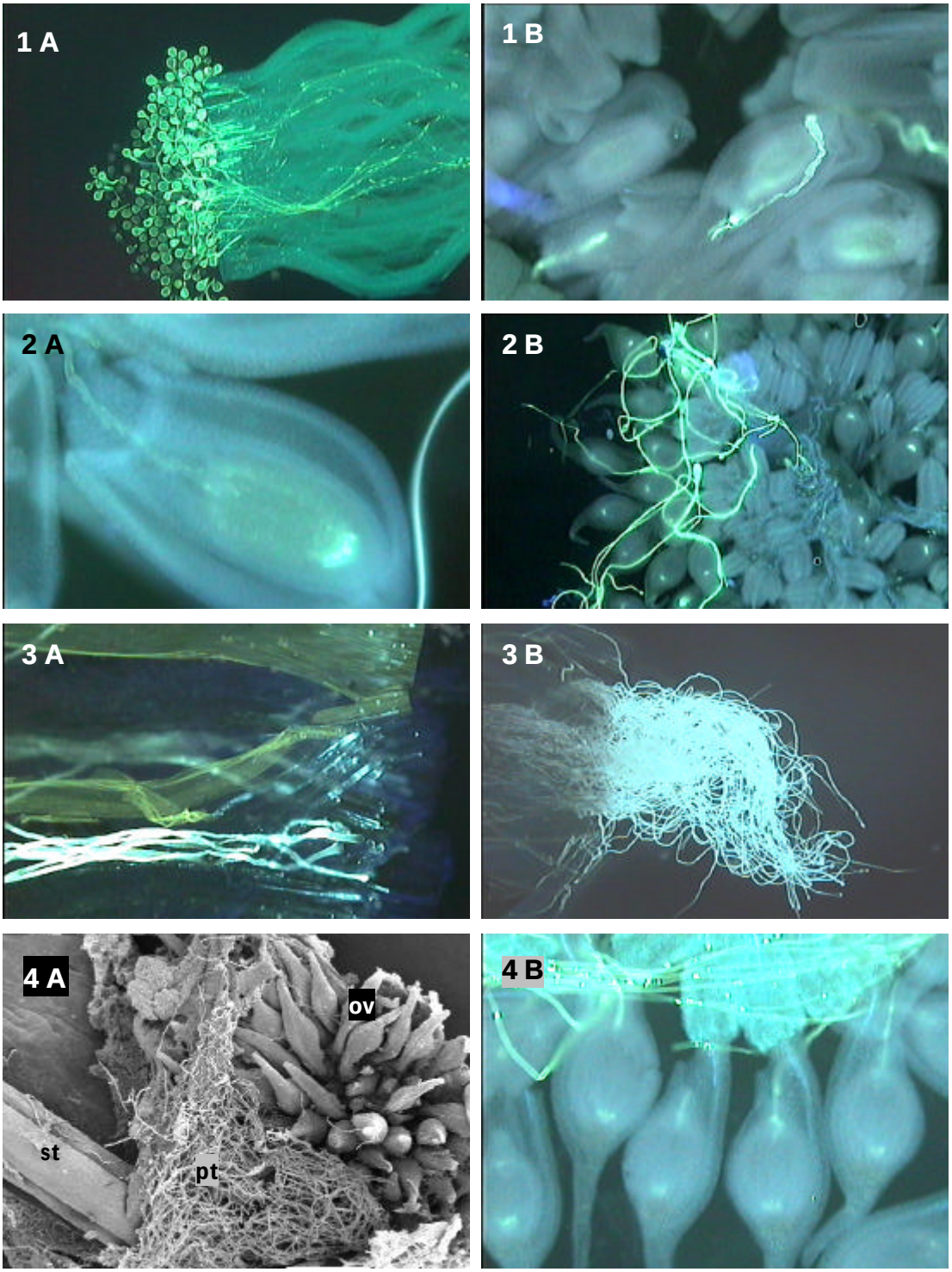
	hours *in vivo*	Style length (cm)	Average number of pollen tubes	Average length of pollen tubes out of style (µm)
Aechmea A	0	1.5	14 ± 23	2258 ± 915
	6	1.5	43 ± 27	2653 ± 839
Vriesea A	0	4	0 ± 0	0 ± 0
	32	4	31 ± 15	1030 ± 411
Vriesea C	0	1-2-5.5	0 ± 0	0 ± 0
	28	3.4	0 ± 0	0 ± 0
Guzmania A	0	4	0 ± 0	0 ± 0
	24	3.6	23 ± 15	1388 ± 592

Mean ± SE (n = 30)

Table 4. Percentage styles where pollen tubes protrude the cut style, % ovules fertilized, fertilization index (% ovaries where one or more ovules are fertilized), % fertilization when only fertilized ovaries are considered, after placental grafted style at anthesis of intercompatible *Aechmea* A (styles *semi in vivo* pollinated 6h and 1.5 cm)

Period	% styles where pollen tubes protrude	% ovules fertilized	FI	% ovules fertilized of FI
Apr 2000	75	8 ± 15 b	37	19 ± 19
Jun 2000	60	2 ± 5 b	24	10 ± 8
Jul 2000	83	16 ± 31 a	30	43 ± 37

a, b: Duncan's multiple range test (Pr = 0.05); mean ± SE (n = 30)



Figures

1. A. Pollen germination on style of *Aechmea* A (4x10), B. Pollen tube grows in the integument of *Vriesea* C after cut style pollination (10x10)
2. A. Pollen tube penetration of the micropyle after placental self pollination of *Tillandsia* A (20x10), B. Placental pollination of *Aechmea* A (4x10)
3. A. Pollen tubes at the stylar end of *Vriesea* C (10x10), B. Pollen tubes emerge from the stylar end after *semi in vivo* pollination of *Aechmea* A (4x10)
4. A. Placental grafted style pollination of *Aechmea* A (st: style, pt: pollen tubes, ov: ovules) B. Pollen tube penetration of ovules after placental grafted style pollination of *Aechmea* A (4x10)