Pollination Timing and Hybrid Seed Production of *Neoregelia*

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Abstract. *Neoregelia* cultivars have been used in many areas for landscaping and indoors in a variety of creative ways, but scientific reports of their pollination and hybridization are presently limited. Cross-combinations of *Neoregelia* cultivars were created to define conditions for pollination timing and to evaluate cross-compatibility. *Neoregelia* cultivars have short-lived flowers. Hybrid seeds were obtained only when cross-pollination was performed before 1200 HR. Results of 19 cross-combinations including six reciprocal crosses revealed that hybrid seeds were obtained in the female parents with a 1.9- to 2.0-cm style length, but not in those with a 2.6- to 3.0-cm style length. The pollen tube penetrated the ovule as early as 1 day after pollination in the compatible cross, whereas swollen pollen tubes were observed at half and two-thirds of the style in the incompatible cross. Removal of 50% of the style length of the female parents could overcome the fertilization barrier for those incompatible crosses and hybrid seeds could be successfully obtained.

Bromeliads have been widely used for potted flowers or landscape decoration for decades, and they are currently considered among the most valuable ornamental plants (Negrelle et al., 2012). Interspecific and intergeneric hybridizations have been made to develop new cultivars in several bromeliad genera, including *Aechmea*, *Guzmania*, *Neoregelia*, *Tillandsia*, and *Vriesea* (Parton et al., 2001; Smith, 1983; Vervaeke et al., 2001). *Neoregelia* cultivars have been popular because of their attractive multicolored foliage, even when the plants are not flowering. Therefore, they have been used in many areas for landscaping and indoor decorations. However, the cultivars that are of interest are hybrids.

Information regarding pollination timing is crucial for successful hybridization because some bromeliads have short-lived flowers and anthesis occurs at a particular time of the day (Foster, 1996; Souza et al., 2016). Foster (1958) stated that most *Neoregelia* flowers open and last only a few hours. Parton et al. (2001) showed that stigma receptivity was high at anthesis and then became significantly lower in *Aechmea*. Souza et al. (2016) showed higher stigma receptivity at anthesis for *Aechmea*, *Alcantarea*, *Ananas*, and *Vriesea*, with the highest

receptivity at 0800 HR; receptivity remained until 1200 HR and declined thereafter.

A prefertilization barrier has been reported for interspecific pollination of *Aechmea*, in which abnormal pollen tubes occur either in the stigma or during penetration of the micropyle (Souza et al., 2017). Prefertilization barriers were observed when the female parents have a longer style than the male parents for hybridization of bromeliads (Parton et al., 2001; Souza et al., 2017; Vervaeke et al., 2001). The cut style technique combined with in vitro culture of the fertilized ovules can lead to the successfully

attainment of intergeneric offspring (Vervaeke et al., 2002, 2003). The lack of seed formation in *Vriesea* and failure of seed germination in *Aechmea* occur despite fertilization, thus characterizing postfertilization barriers (Parton et al., 2001). Little information is available regarding the fertilization barriers of crossing *Neoregelia* species and/or cultivars.

The objectives of the present study were to 1) define the conditions for pollination timing, and 2) to observe whether the parental style length can affect seed formation when *Neoregelia* cultivars are crossed, and if so, if the cut style technique can overcome the fertilization barrier.

Materials and Methods

Plant materials and culture. Suckers of *Neoregelia* cultivars with six to eight leaves were removed from the stock plants after flowering and planted in plastic containers containing 1.2 L of coconut husk chips commonly used for the commercial production of bromeliads. Plants were grown in a glasshouse with an average noontime light intensity of 400 to 900 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and temperature of 25 to 35 °C. Plants were fertilized monthly with water-soluble 20N–8.7P–16.6K (20–20–20 General Purpose; JR Peters, Allentown, PA) at 1 g·L⁻¹.

The corolla and anthers were removed in the female parent before the corolla lobes unfolded. Fresh pollen was always collected at anthesis and used immediately for cross-pollination from March to May, when natural flowering occurred, between years 2008 and 2018. Three to four months after pollination, the seeds were collected, counted, and sown in 9-cm-diameter plastic pots containing coconut husk chips at 25 to 30 °C and 90% to

Table 1. Effects of pollination time on seed number per fruit and seed germination of *Neoregelia* cross-combinations.

Crosses (cross no.)	Pollination time ^z	Seed number per fruit	Seed germination (%)
Gold Fever × Pink Sensation (1)	0700–0950 HR	306.7 a ^y	10.1
	1000–1250 HR	155.0 b	60.7
	1300–1550 HR	0 c	—
	1600–1800 HR	0 c	—
Gold Fever × Topaz (2)	0700–0950 HR	291	82.4
	1300–1550 HR	0	—
		*	
Gold Fever × Perfecta Tricolor (3)	0700–0950 HR	368	64.9
	1300–1550 HR	0	—
		*	
Gold Fever × Puerto Rico (4)	0700–0950 HR	160	83.0
	1300–1550 HR	0	—
		*	
Little Rose × Deroose's Medusa (5)	0700–0950 HR	56	19.6
	1300–1550 HR	0	—
		*	
Little Rose × Gespacho (6)	0700–0950 HR	162	22.8
	1300–1550 HR	0	—
		*	
Little Rose × Topaz (7)	0700–0950 HR	214	22.4
	1300–1550 HR	0	—
		*	

^zCorolla lobes unfolded at 0700–0950 HR, flowers were at anthesis at 1000–1250 HR, corolla lobes and stigma curling occurred at 1300–1550 HR, and flowers wilted at 1600–1800 HR.

^yMean separation within column by least significant difference at $P < 0.05$ level.

*Significant at $P < 0.05$ by t test.

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100% relative humidity in a greenhouse. Seedling numbers and germination percentages were recorded at 2 weeks after sowing.

The origins of cultivars used in this study are as follows. 'Deroose's Medusa' is a spineless offspring selected from *N. carolinae* 'Meyendorffii'. 'Perfecta Tricolor' is a striped offspring selected from *N. carolinae* 'Marechalii'. 'Skotak's Orange Crush' is a hybrid derived from *N. carolinae* with an unknown male parent. 'Charm' is a hybrid derived from crossing *N. marmorata* and *N. chlorosticta*. 'Gespacho' is a hybrid derived from crossing *N. chlorosticta* and *N. 'Fireball'*. 'Gold Fever' is a hybrid derived from crossing *N. 'Cinnabar'* with an unknown male parent. 'Little Rose' is a hybrid derived from crossing *N. chlorosticta* with an unknown male parent. Both 'Pink Sensation' and 'Puerto Rico' are hybrids derived from crossing *N. carolinae* with unknown male parents. 'Topaz' is a hybrid derived from crossing *N. 'Orange Delight'* and *N. olens*. 'Orange Delight' is a hybrid derived from *N. carolinae*.

Expt. 1: Pollination timing. Fresh pollen of 'Pink Sensation' was carefully transferred to the stigma of 'Gold Fever' using forceps. Pollination was performed on sunny days at 0700–0950 HR (corolla lobes unfolded), 1000–1250 HR (flowers at anthesis), 1300–1550 HR (corolla lobes curling inwards, stigma curling), and 1600–1800 HR (flowers wilted). More cross-pollination was performed at 0700–0950 HR and 1300–1550 HR. The combinations included 'Gold Fever' as the female parent crossed with 'Topaz', 'Perfecta Tricolor', and 'Puerto Rico', and 'Little Rose' as the female parent crossed with 'Deroose's Medusa', 'Gespacho', and 'Topaz'. Each pollination timing consisted of three replicates. Seed number per fruit, expressed as an average of the replicates, was counted. Seed germination was recorded 2 weeks after being sown in a glasshouse at 25 to 30 °C under light intensity conditions of 400 to 900 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. A comparison between different pollination timing was performed using the least significant difference or *t* test using CoStat 6.4 (CoHort Software, Monterey, CA).

Expt. 2: Cross-combinations between cultivars with different style lengths. Thirteen cross-combinations were made, including cultivars Perfecta Tricolor, Deroose's Medusa, Meyendorffii, and Skotak's Orange Crush with a long style length (2.6–3.0 cm) and cultivars Gold Fever, Gespacho, and Charm with a short style length (1.9–2.0 cm). Thirty styles were measured from each cultivar to determine the average style length. An additional six reciprocal crosses were designed to determine whether the style length of the female parent could affect seed production. These include crosses between 'Gespacho' and 'Deroose's Medusa', 'Gold Fever' and 'Deroose's Medusa', and 'Gold Fever' and 'Perfecta Tricolor'. Each combination consisted of three to five replicates. Seed number per fruit, expressed as an average of the replicates, was counted.

In vivo pollen tube growth was observed following the methods described by Kho and Baër (1965) at 5 d after pollination in 'Skotak's Orange Crush' $\times$ 'Gespacho' and at 1 d after pollination in 'Gespacho' $\times$ 'Deroose's Medusa'. Samples of pollinated gynoecium were taken, fixed with 75% ethanol, and stored at 4 °C. Then, samples were

rinsed thoroughly with distilled water and softened with 1 N sodium hydroxide for 30 min at 65 °C until semi-transparent. After being rinsed with distilled water, samples were stained with 0.025 M aniline blue (Waldeck, Münster, Germany) solution (in 1 M tripotassium phosphate) for 1 h. Samples were observed with a microscope (Nikon

Fig. 1. Pistil of *Neoregelia* 'Meyendorffii' with a long style (left) and pistil of 'Gold Fever' with a short style (right). Bar = 1 cm.

Eclipse E600; Nikon, Tokyo, Japan) equipped with a fluorescent lamp (450–490 nm) and a charge-coupled device digital camera (Optonics Microfire True Color Firewire Digital CCD; Meyer Instrument, Houston, TX). Each sample consisted of three to five replicates, and each replicate consisted of one gynoecium.

Expt. 3: Cut-style pollination. To overcome possible pollination barriers, we performed cut-style pollination following the methods described by Vervaeke et al. (2002, 2003). The upper 50% of the style length of the female parents was cut and removed with sharp scissors. Cross-combination included ‘Perfecta Tricolor’ × ‘Gold Fever’, ‘Skotak’s Orange Crush’ × ‘Gold Fever’, and ‘Meyendorffii’ × ‘Gold Fever’. Each combination consisted of three to five replicates. Seed number per fruit, expressed as an average of the replicates, was counted. A comparison of seed number was performed between intact styles (control) and styles with 50% of the length removed by using the *t* test (CoStat 6.4; CoHort Software, Monterey, CA).

Results and Discussion

Expt. 1: Pollination timing. Emasculated flowers of ‘Gold Fever’ were cross-pollinated with ‘Pink Sensation’ from 0700 to 1800 HR. Results showed that more seeds but lower germination percentages were observed when pollinated between 0700 and 0950 HR compared to between 1000 and 1250 HR (Table 1). Pollination after 1300 HR resulted in no seed production (cross 1). Results from another six crosses (crosses 2–7) revealed that seeds were obtained when pollinated between 0700 and 0950 HR as compared with no seed production when pollinated between 1300 and 1550 HR.

Our results indicate that successful pollination timing is rather short and that pollination should be performed before 1200 HR for *Neoregelia* hybridization (Table 1). The flowers of some bromeliad species have a rather short opening and receptivity time-frame (Foster, 1958; Souza et al., 2016). Our previous unpublished investigations showed that *Neoregelia* pollen sampled 1 d after anthesis had a 20% to 70% germination rate when cultured in BK medium (Brewbaker and Kwack, 1963) containing 10% sucrose. It was probable that no seed set would result from a short receptivity time; instead, pollen inviability resulted when cross-pollination was performed after 1200 HR.

Expt. 2: Cross-combinations between cultivars with different style lengths. *Neoregelia* have a dry stigma with multiseriate papillae, and cultivars differ in their style length (Heslop-Harrison and Shivanna, 1977) (Fig. 1; Table 2). Crosses between cultivars resulted in no seed production from female parents, with the style length ranging from 2.6 to 3.0 cm (crosses 8–14). In contrast, 75 to 542 seeds per fruit were obtained from female parents with 1.9- to 2.0-cm style lengths (crosses 15–20). Parton et al. (2001) observed

that differences in the style length of the female and male parents can influence seed production for other Bromeliaceae. Our results suggest that seed production appeared to be related to the style length of female parents for *Neoregelia* hybridization.

Reciprocal crosses were made to determine whether the maternal style length could affect seed production (Table 3). Results

showed that seeds were obtained only in female parents with a 1.9-cm style length, whereas no seed production occurred in female parents with 2.6- to 2.8-cm style length (crosses 21–26). Vervaeke et al. (2001) also reported unilateral congruity between *Aechmea* due to differences in style length.

At 5 d after pollination, swollen pollen tubes were observed in half to two-thirds of

Table 2. Seed number obtained from *Neoregelia* cross-combinations between cultivars with different style lengths.

Female parent	Male parent	Cross no.	Style length (cm)		Seed number
			Female	Male	
Perfecta Tricolor	Skotak’s Orange Crush	8	2.8	2.7	0
Deroose’s Medusa	Perfecta Tricolor	9	2.6	2.8	0
Meyendorffii	Gold Fever	10	3.0	1.9	0
Meyendorffii	Gespacho	11	3.0	1.9	0
Skotak’s Orange Crush	Meyendorffii	12	2.7	3.0	0
Skotak’s Orange Crush	Gold Fever	13	2.7	1.9	0
Skotak’s Orange Crush	Gespacho	14	2.7	1.9	0
Gold Fever	Gespacho	15	1.9	1.9	233.2
Gold Fever	Charm	16	1.9	2.0	187.0
Gespacho	Gold Fever	17	1.9	1.9	203.0
Charm	Deroose’s Medusa	18	2.0	2.6	442.0
Charm	Meyendorffii	19	2.0	3.0	542.0
Charm	Gespacho	20	2.0	1.9	493.0

Table 3. Seed number obtained from reciprocal crosses between *Neoregelia* cultivars with different style lengths.

Cross-combination	Cross no.	Style length (cm)		Seed number
		Female	Male	
Gespacho × Deroose’s Medusa	21	1.9	2.6	89.5
Deroose’s Medusa × Gespacho	22	2.6	1.9	0
				*
Gold Fever × Deroose’s Medusa	23	1.9	2.6	93.0
Deroose’s Medusa × Gold Fever	24	2.6	1.9	0
				*
Gold Fever × Perfecta Tricolor	25	1.9	2.8	368.0
Perfecta Tricolor × Gold Fever	26	2.8	1.9	0
				*

*Significant at *P* < 0.05 by *t* test.

Fig. 2. The abnormal swollen pollen tubes in half and two-thirds of the style in *Neoregelia* ‘Skotak’s Orange Crush’ crossed with ‘Gespacho’ 5 d after pollination (A). Pollen tubes penetrated the micropyle in ‘Gespacho’ crossed with ‘Deroose’s Medusa’ 1 d after pollination (B). Arrow indicates pollen tubes. Bar = 200 µm.

Table 4. Effect of the removal of 50% of the style length of the female parent on seed number of three *Neoregelia* cross-combinations.

Crosses (cross no.)	Style ^z	Seed number
Perfecta Tricolor × Gold Fever (27)	–	0.0
	+	227.3 *
Skotak's Orange Crush × Gold Fever (28)	–	0.0
	+	125.0 *
Meyendorffii × Gold Fever (29)	–	0.0
	+	25.0 *

^zIntact style (–) or removal of 50% of the style length (+).

*Significant at $P < 0.05$ level by t test.

the style in the 'Skotak's Orange Crush' × 'Gespacho' cross (Fig. 2A), which may explain why this combination did not produce any seeds (cross 14) (Table 2). A swollen pollen tube and callose deposition at the tube tip have been documented in intraspecific, interspecific, and intergeneric crosses, even when the cross seemed to be congruent (Parton et al., 2001; Souza et al., 2017). Deposition of the callose influenced the maintenance of turgor pressure and osmotic balance in the pollen tube. Regular callose deposition can serve as a mechanical barrier preventing plasma at the apex from flowing backward or preventing the pollen tubes from shrinking (Souza et al., 2017). However, there is a close relationship between incompatibility and callose deposition on pollen tubes. For example, an abnormally increased deposition of callose resulted in incompatible swollen pollen tubes bursting within the style in *Petunia × hybrida* (Ünal et al., 2013). We suggest that the incompatibility in the 'Skotak's Orange Crush' × 'Gespacho' cross was due to the rejection reaction of the pollen tube and style. In contrast, pollen tubes had penetrated the ovule as early as 1 d after pollination in 'Gespacho' × 'Deroose's Medusa' (Fig. 2B), which led to seed production (cross 21) (Table 3).

Expt. 3: Cut-style pollination. Removal of 50% of the style length of the female parents resulted in seed production. No seed was obtained from the female parents with an intact long style (Table 4). These results are consistent with previous observations that the

style length of the female parents could affect successful cross-pollination (Tables 2 and 3).

Several techniques that can be used to overcome reproductive barriers in Bromeliaceae were described by Vervaeke et al. (2002), such as cut-style pollination, placental grafted-style pollination, and placental pollination. The present study showed that cut-style pollination could bypass the pollen-style rejection reaction in the incompatible crosses in *Neoregelia* and obtain hybrid seeds. The cut-style techniques have been used successfully to develop intergeneric hybrids of *Aechmea* (Vervaeke et al., 2002, 2003).

Conclusions

Neoregelia cultivars have short-lived flowers; therefore, cross-pollination should be performed before 1200 HR. Crosses between *Neoregelia* cultivars may have prefertilization barriers confined within the style, as reported previously for other Bromeliaceae (Vervaeke et al., 2001, 2002). We suggest using female parents with a short style for successful cross-pollination because unilateral incongruity appeared to be related to the female style length. Removal of 50% of the style length of female parents could overcome the prefertilization barrier and obtain hybrid seeds.

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