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Rain pollination provides reproductive assurance in a deceptive orchid

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- *Background and Aims* Abiotic pollination by wind or water is well established in flowering plants. In some species pollination by rain splashes, a condition known as ombrophily, has been proposed as a floral strategy. However, evidence for this type of abiotic pollination has remained controversial and many reported cases have subsequently been shown to be false. This study investigates ombrophily in the deceptive orchid *Acampe rigida* to determine the mechanism by which this species is able to maintain high fecundity, despite flowering during the rainy season in south-west China when pollinators are scarce.
- *Methods* The floral mechanisms promoting rain pollination in *A. rigida* were observed and described in detail. Controlled pollination experiments and observations of floral visitors were conducted. A field experiment using rain shelters at 14 sites in Guangxi, south-west China, evaluated the contribution of rain pollination to fruit-set.
- *Key Results* During rainfall, raindrops physically flicked away the anther cap exposing the pollinarium. Raindrops then caused pollinia to be ejected upwards with the strap-like stipe pulling them back and causing them to fall into the stigmatic cavity, resulting in self-pollination. Neither flower nor pollen function were damaged by water. Although *A. rigida* is self-compatible, it is incapable of autonomous self-pollination without the assistance of rain splashes. The results of the rain-sheltering experiment indicated that rain pollination contributed substantially to increasing fruit-set, although there was variation among sites in the intensity of this effect.
- *Conclusions A. rigida* flowers during the rainy season, when pollinators are scarce, and ombrophily functions to provide reproductive assurance without compromising opportunities for outcrossing.

Key words: Abiotic pollination, *Acampe rigida*, floral adaptation, ombrophily, rain-mediated self-pollination, deceptive orchid.

INTRODUCTION

Abiotic pollination refers to pollination mediated without the involvement of animals, but excluding self-pollination. Anemophily (pollination by wind) and hydrophily (pollination by water) are the two principal forms of abiotic pollination and occur in approx. 20 % of angiosperm families (Ackerman, 2000). Hagerup (1950) described an unusual form of pollination in several species in the windswept and rainy conditions characteristic of the Faroe Islands. Flowers that remained open during rain filled with water enabling pollen to be transported to stigmas, resulting in self-pollination. Pollination mediated by rain splashes (ombrophily – Daumann, 1970) can also occur and such rain-mediated pollination mechanisms have generally been regarded as strategies for reproductive assurance functioning to promote self-pollination under adverse conditions when pollinators are infrequent (Stebbins, 1957; Fægri and van der Pijl, 1979). However, because rain can damage flowers and reduce pollen viability (Daumann, 1970; Mao and Huang, 2009), the extent to which rain pollination represents an adaptive floral strategy remains unclear. Indeed, several of the cases reported by Hagerup (1950) as exemplifying this form of abiotic pollination have subsequently been shown to be false, or are open to alternative interpretation (e.g. Daumann, 1970; Lundqvist, 1992; Galen *et al.*, 1993; Jacquemart, 1996).

Fruit-set in many species of orchids is chronically pollen-limited owing to infrequent pollinator visitation (Tremblay *et al.*, 2005). This situation is particularly common in species that employ deceit pollination and provide no floral rewards, a common condition occurring in about one-third of the family (Dressler, 1990). Floral deception may be beneficial when pollinators are abundant, but if pollen limitation persists the evolution of nectar rewards, or a shift to autonomous self-pollination, might be expected over longer timescales (Jersáková *et al.*, 2006). Although a remarkable diversity of pollination systems are represented in the Orchidaceae, no species has been reported that is exclusively abiotically pollinated (Tremblay *et al.*, 2005).

Acampe comprises about ten epiphytic or lithophytic species distributed from tropical and subtropical Africa through the Himalayan region and Indochina to South-East Asia (Chen and Wood, 2009). Among the four species occurring in China, *A. rigida* is most common and is widely distributed in karst regions between 300 and 1800 m in the Provinces of Yunnan, Guangxi, Guangdong and Hainan. Little is known about the pollination biology of *Acampe*, but *A. rigida* is a typical deceptive orchid with small attractive flowers that are fragrant but rewardless (Fig. 1A). Plants often form dense colonies of shoots and it is difficult to determine with any certainty whether individual flowering shoots represent distinct

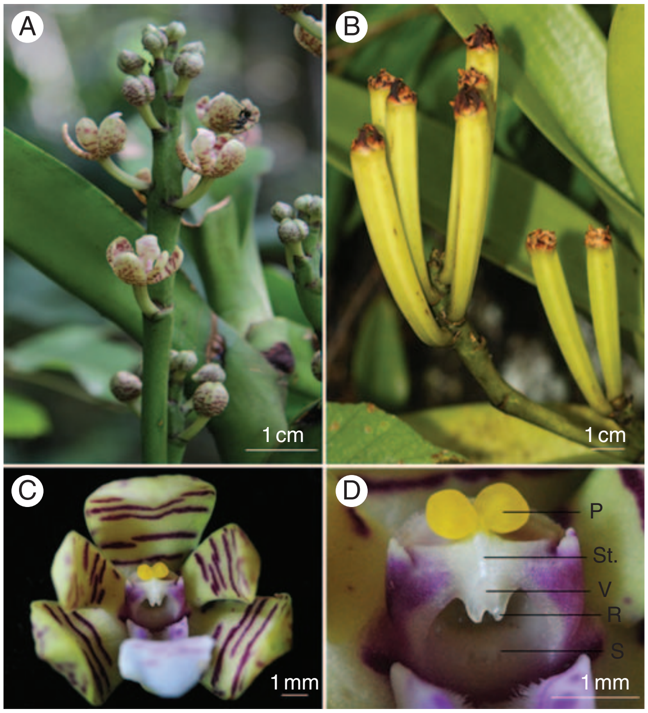

FIG. 1. Reproductive features of the deceptive orchid *Acampe rigida*. (A) Inflorescence with vertically orientated, bowl-shaped, spirally arranged flowers. (B) Infructescence illustrating high fruit-set. (C) Flower with fleshy sepals and petals. (D) The structure of the pollinarium and stigma: P, pollinia; St, stipe; V, viscidium; R, rostellum; S, stigmatic cavity.

genets. Our preliminary field observations of this species indicated that unlike most deceptive orchids the fruit-set of populations is relatively high (Fig. 1B), despite flowering during a period of the year (the middle of August to the end of September) when rainfall is frequent, and opportunities for insect-mediated cross-pollination are often very limited. These observations motivated us to investigate the floral biology of the species in an effort to determine the mechanisms that might be responsible for maintaining high fruit-set.

MATERIALS AND METHODS

Floral traits and observations

To determine the flowering period of *Acampe rigida* we monitored plants weekly from 2007 to 2010 in the Xishuangbanna National Nature Reserve (XNNR, 21°41′N, 101°25′E; 580 m a.s.l.) in Yunnan, south-west China. We obtained precipitation data from the Xishuangbanna Tropical Rainforest Ecosystem Station located 2 km north-west of our study sites to establish if flowering occurred during the rainy season. To obtain information on floral display and the longevity of flowers we counted total flower number on 30 randomly selected inflorescences from different plants, and floral longevity was measured for 30 flowers, each of which was randomly selected from among the 30 inflorescences.

During the flowering seasons of 2009 and 2010, we marked 36 flowers on 21 inflorescences from 17 individuals to observe and video the process of rain pollination at XNNR. To investigate pollinia removal and deposition after one significant natural rainfall event we randomly selected 101 flowers distributed among 17 plants. After rain we checked all flowers to determine if any of the floral parts were obviously damaged. To assess the effect of rain on pollen function, we hand-pollinated nine flowers with pollinia that had been submersed in rainwater for 36 h and measured fruit-set. We investigated the effects of simulated rain on pollination and fruit-set in the laboratory using six potted plants of *A. rigida*. We used a showerhead to mimic raindrops and allowed these to fall from heights of up to 1 m on flowers.

We monitored insect visitors to flowers of *A. rigida* for approx. 132 h at three locations at XNNR to determine the extent to which pollinators are important for pollination. At site 1 observations were made between 0820 and 1800 h from 25 to 29 August and on 9 September in 2009; at sites 2 and 3 between 0830 and 1730 h from 23 to 25 August and on 2, 3, 6 and 9 September in 2010; and also between 0900 and 1600 h on 20–23 August in 2010. The days we chose were sunny when we started observations in the morning, but on most days there were typically one or more periods of heavy afternoon rain during which we still continued observations. During the flowering season of 2010 on eight rainless days we also monitored 127 newly opened flowers on three plants at three times during the day (0830, 1500 and 1800 h) to check if pollinia removal and deposition had occurred.

Hand-pollination experiments

To investigate the compatibility status and capacity for autonomous self-pollination in *A. rigida*, four pollination treatments were conducted on randomly selected flowers in 2009 and 2010 at the Wild Orchid Collection, which is located in the protected area of Xishuangbanna Tropical Botanical Garden (XTBG) and is 1·5 km away (north-west) from our study sites at XNNR. The treatments were: (1) self-pollination: 12 and 42 flowers from three and 12 plants were hand-pollinated with pollinia from the same flower in 2009 and 2010, respectively; (2) cross-pollination: 56 and 47 flowers from five and 13 plants were hand cross-pollinated with the pollinia from other individuals in 2009 and 2010, respectively; (3) bagged and with rain-shelter: 116 flowers on 20 inflorescences from ten plants were bagged and rain-sheltered by using plastic shelters positioned 30 cm above the inflorescences during flowering in 2010; (4) control: 101 and 337 flowers from 17 and 13 plants were marked for natural open pollination in 2009 and 2010, respectively. We analysed differences in fruit-set among treatments, excluding the bagged treatment (3), which set no fruit, with a generalized linear mixed model (SAS GLIMMIX Procedure: SAS Institute Inc., 2011) that considered fruit-set as a binary response (logit link function: Fitzmaurice *et al.*, 2004). Pollination treatment was treated as a fixed factor and year as a random factor. We conducted two specific contrasts: hand self-pollination (1) versus hand cross-pollination (2) to test for the presence of self-incompatibility, and supplemental pollinations (1 and 2) versus open-pollination (4) to evaluate the extent to which natural fruit-set might be pollen-limited. We pooled both supplemental pollinations because fruit did not differ between them (see Results). Our analysis accounts for the lack of independence associated with the measurement of multiple flowers per plant with a compound-symmetric variance–covariance matrix, which was used to adjust the denominator degrees of freedom according to the method of Kenward and Roger (1997). In reporting the results of this experiment we presented back-transformed least-squares means, which are associated with asymmetric standard errors.

To determine the viability of seeds from self- versus cross-pollination, capsules from the hand self- and cross-pollination treatments in 2009 were harvested just before capsules split open in March 2011. We then conducted TTC (2,3,5-triphenyl tetrazolium chloride) tests to determine the viability of non-germinated seeds, as used previously for other orchid species (Vujanovic *et al.*, 2000). In addition, seeds were examined microscopically to determine if embryo development had occurred, and seeds were germinated *in vitro* on MS medium (Murashige and Skoog, 1962).

Rain-shelter experiment

To evaluate the contribution of rain pollination to fruit-set under field conditions we conducted a rain-shelter experiment on 236 inflorescences from 21 individuals at 14 sites in Guangxi Autonomous Region of south-west China during 1–14 August 2010 (Table 1). At each site we randomly assigned inflorescences to one of two groups, a rain-shelter treatment ($n = 95$), in which the shelters prevented raindrops from contacting the flowers, and an open-pollinated (unmanipulated) treatment ($n = 141$), in which inflorescences were unprotected from rain. As above, inflorescences in the rain-shelter treatment were covered with plastic sheets (up to 1 × 2 m in size) positioned at least 30 cm above the tallest inflorescence. The shelters were attached to tree branches or shrubs by rope. The shelters were made of translucent plastic and were open on all sides allowing free circulation of air and access to inflorescences by pollinators. Frequent run off from rain ensured that the substrates on which plants in the sheltered treatment were growing were moist and plants were not water-stressed during the experiment. During 25–31 October we recorded the fruit-set of inflorescences in each treatment. As in the controlled pollination experiment, we analysed differences in fruit-set between the open-pollinated and rain-shelter treatments with a generalized linear mixed model (SAS GLIMMIX Procedure: SAS Institute Inc., 2011) that considered fruit-set as a binary response (logit link function: Fitzmaurice *et al.*, 2004). Treatment was treated as a fixed factor, sites as a random factor. This analysis also accounts for the lack of independence associated with the measurement of multiple flowers per plant.

RESULTS

The mechanism of rain-pollination

The inflorescences of *A. rigida* have a total of 13 ± 8·05 (mean ± s.d.; range 1–37) vertically orientated bowl-shaped flowers that are spirally arranged (Fig. 1A). Sepals and petals are thick and fleshy and the column is positioned in the centre of the flower (Fig. 1C). During rain, raindrops served to physically flick the anther cap away, thereby exposing the pollinarium. The structure of the pollinarium involves a viscidium held in place by a biforked rostellum with stretchable stipes linking the two yellow pollinia with the viscidium. These structures are reminiscent of a ball joined to a rubber cord (Fig. 1D). During rain the pollinia are bounced upward by raindrops and are then pulled back by the stipes, resulting in the pollinia falling in a precise way into the stigmatic cavity. In a sample of 101 flowers distributed among 17 plants that were exposed to rain under field conditions, we found that in 70 % the pollinia had been ejected and in 60 % the pollinia were subsequently deposited on the stigmas. The same pollination process was also observed in the laboratory when we used a showerhead to mimic raindrops and this resulted in high levels of fruit-set. We video recorded pollination by rain in the field and this is available at the XTBG website (http://ecrg.groups.xtbg.ac.cn/).

Table 1. *Locations, sample size and mean fruit-set of open-pollinated and rain-sheltered plants of* Acampe rigida *at 14 sites in Guangxi, China*

Study site	Location: latitude/longitude/altitude (m)	Open-pollination treatment		Rain-shelter treatment	
		Fruit-set (%, ± s.d.)	Sample size: inflorescence (plant)	Fruit set (%, ± s.d.)	Sample size: inflorescence (plant)
6th village, Mashan	23°40′157″N/108°11′075″E/205	8·4 ± 3·4	5 (1)	0	3 (1)
Xinhan, Mashan	23°37′048″N/108°12′510″E/214	14·4 ± 10·8	13 (1)	9·4 ± 7·64	6 (1)
Ganchang, Bama	24°15′924″N/107°14′408″E/465	24·5 ± 17·4	3 (1)	4·3 ± 8·1	8 (1)
Banshe, Bama	24°12′560″N/107°11′478″E/354	23·3 ± 12·2	34 (2)	0	30 (2)
Yanglv, Tianyang	23°36′346″N/106°50′094″E/450	32·4 ± 7·0	4 (1)	0	3 (1)
Liangwang1, Tianyang	23°36′601″N/106°49′709″/469	15·0 ± 7·1	2 (1)	12·1 ± 3·0	2 (1)
Liangwang2, Tianyang	23°36′601″/106°49′709″E/459	38·9 ± 10·7	6 (1)	3·7 ± 4·3	4 (1)
Longhua1, Tianyang	23°32′945″N/106°48′799″E/771	35·6 ± 34·6	2 (1)	0	3 (1)
Longhua2, Tianyang	23°32′878″N/106°48′354″E/806	45·1 ± 16·8	4 (1)	0	1 (1)
Hurun, Jingxi	23°00′044″N/106°37′077″E/609	17·1 ± 13·1	24 (2)	8·4 ± 10·6	11 (2)
Jingxi	23°14′434″N/106°02′359″E/884	29·7 ± 12·0	17 (1)	1·8 ± 5·5	13 (1)
Qugang, Jingxi	23°10′899″N/106°23′734″E/828	41·5 ± 19·6	17 (1)	2·4 ± 3·4	2 (1)
Bumin, Longzhou	22°30′591″N/106°46′210″E/355	11·0 ± 12·9	4 (1)	2·1 ± 3·6	3 (1)
Pingxiang, Longzhou	22°07′127″N/106°44′775″E/268	17·2 ± 9·5	6 (1)	12·2 ± 17·4	6 (1)

We observed no damage to flower parts after heavy rain, and the fruit-set of flowers hand-pollinated with pollinia submersed in rain water for 36 h was 100 % ($n = 9$ flowers), indicating that rain does not appear to damage pollen function. At XNNR, where our study was conducted, the rainy season is from May to October, and the flowering period of *A. rigida* is from August to September. The maximum rainless period during this time is 5 d, and the floral longevity of *A. rigida* is 7 ± 3 d ($n = 30$). Thus, flowers of *A. rigida* typically receive at least one period of significant rainfall and more commonly many more.

Hand-pollination experiments and observations of insect visitors

The overall effect of pollination treatment on fruit-set was highly significant ($F_{3,3\cdot2} = 16\cdot04$, $P = 0\cdot021$); however, there was little variation among the two years for each of the three treatments, and this was reflected in the lack of a 'year effect' in the statistical analysis ($t = 0\cdot131$, d.f. $= 1$, $P = 0\cdot657$). There was no significant difference between the fruit-set of supplemental cross- and self-pollinations ($F_{1,60} = 1\cdot74$, $P = 0\cdot192$), with both pollination treatments yielding similarly high levels of fruit-set (Fig. 2). This indicates that *A. rigida* is strongly self-compatible. Seeds from both self- and cross-pollination stained approx. 100 % following TTC tests and well formed embryos were evident when viewed microscopically. Protocorms established from both seed types after they were sown on MS media for 40–50 d.

Flowers that were bagged but sheltered from rain set no fruit. Natural levels of fruit-set in open-pollinated flowers were sub-maximal in each year (2009: 39·6 %, $n = 101$; 2010: 43 %, $n = 337$) and supplemental pollinations increased fruit-set significantly compared with open-pollinated flowers ($F_{1,60} = 55\cdot17$, $P < 0\cdot001$, Fig. 2). This suggests that flowers were pollen limited and is consistent with observations of the chronic paucity of insect visitation during the 2009 and 2010 flowering seasons. During field observations totalling approx. 132 h only a single insect visit to a flower by a male scoliid wasp (*Scolia* sp.) was recorded. In 2010, flowers monitored during the rainless period ($n = 38$) showed no evidence of insect visitation, as their pollinia remained intact, indicating that no effective pollination occurred despite fine weather.

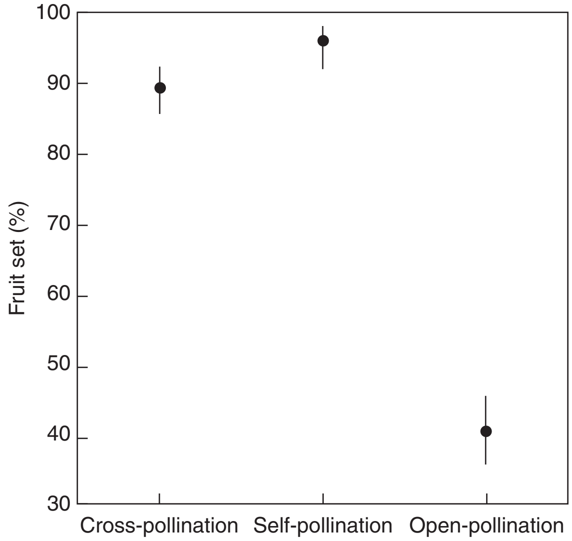

FIG. 2. Mean fruit-set of *Acampe rigida* flowers following three pollination treatments: supplemental cross- and self-pollination and open-pollination, conducted in 2009 and 2010. The results were not significant between years and are therefore combined (see Results). The least-squares means are back-transformed and are associated with asymmetric standard errors.

Rain-shelter experiment

We conducted a rain-shelter experiment at 14 sites in 2010 to evaluate the importance of rain in contributing towards fruit-set. In open-pollinated flowers exposed to rain, fruit-set was significantly higher than for flowers sheltered from rain (open-pollinated: 23·3 %, lower s.e. = 3·45 %, upper s.e. = 3·85 %, $n = 141$ inflorescences; rain-sheltered: 3·12 %, lower s.e. = 0·98 %, upper s.e. = 1·32 %, $n = 95$ inflorescences; $F_{1,24\cdot9} = 60\cdot26$, $P < 0\cdot0001$; Table 1). In addition, fruit-set varied significantly among sites ($\chi^2 = 7\cdot17$, d.f. $= 1$, $P = 0\cdot0037$). No pollinators were observed visiting flowers at any of the sites during the experiment.

DISCUSSION

Sprengel (1793) first recognized that precipitation during the blooming period of animal-pollinated plants could have potentially harmful effects on reproductive success. Rain may damage sexual organs, reduce the viability of pollen and indirectly limit fecundity by deterring pollinator visits to flowers. The importance of rain as a selective factor on floral traits is thus well recognized and a variety of floral strategies have been proposed that function to limit its deleterious consequences (Darwin, 1876, p. 376; Hagerup, 1950; Aizen, 2003; Mao and Huang, 2009). By contrast, in some species it has been proposed that rain might aid reproduction by facilitating self-pollination when pollinators are in short supply, although experimental evidence to support this suggestion has been weak.

Evidence of rain promoting self-pollination

Hagerup (1950) described rain-assisted pollination in *Caltha palustris*, *Narthecium ossifragum* and several species of *Ranunculus*, although subsequent studies cast doubt on this interpretation. For example, *N. ossifragum* is capable of autonomous self-pollination in the absence of rain and rain has been shown to have deleterious effects on both female fertility and pollen germination (Jacquemart, 1996). Similarly, *Caltha palustris* is now known to be self-incompatible (Lundqvist, 1992), thus preventing rain-mediated self-fertilization. The primary function of the bowl-shaped flowers of many *Ranunculus* spp. appears to be for solar tracking (e.g. Totland, 1994; Sherry and Galen, 1998) rather than aiding in water-mediated self-pollination, although the latter function has not been completely ruled out.

Rain has been suggested as playing a role in the pollination of several species of Orchidaceae. It has been proposed that raindrops increase the incidence of self-pollination in *Liparis loeselii* and *Oeceoclades maculata*, two species also capable of autonomous self-pollination (Catling, 1980; González-Díaz and Ackerman, 1988). *O. maculata* populations from Puerto Rico are nectarless and appear to set fruit entirely by rain-assisted autogamy (González-Díaz and Ackerman, 1988). By contrast, in southern Brazil they produce nectar and are occasionally visited by species of *Heliconius* butterflies, although most fruit-set results from spontaneous and rain-assisted autogamy (Aguiar *et al.*, 2012). In the deceptive *Cyrtopodium polyphyllum*, water accumulates on the stigma, dissolving the adhesive surface and causing a viscous drop which contacts the pollinarium. When this shrinks due to evaporation, the drop moves the pollinarium onto the stigmatic surface resulting in self-pollination (Pansarin *et al.*, 2008). However, the operation of this mechanism does not appear to be very frequent as only 6·5 % of flowers had rain drops accumulating on stigmas and fruit-set resulting from rain assisted self-pollination was estimated to be only 2·2 %, the low value reflecting, in part, the inability of this species to self-pollinate autonomously. Our experimental results suggest that rain-assisted self-pollination contributes significantly to maintaining relatively high levels of fruit-set in natural populations of *A. rigida* and thus appears to be a general feature of the pollination biology of this species. Because *A. rigida* is incapable of autonomous self-pollination, the physical intervention of rain splashes seems to be the only mechanism by which self-pollination can regularly occur.

For ombrophily to function as a pollination mechanism a species must be self-compatible. Several *Acampe* species are reported as self-incompatible, or at least self-sterile (Agnew, 1986), but our study demonstrates that *A. rigida* is fully self-compatible, producing equally viable seeds from self- and cross-pollination. Significantly, despite being self-compatible *A. rigida* does not possess floral mechanisms enabling significant autonomous (spontaneous) self-pollination. Evidence to support this comes from three sources. First, flowers that were bagged and protected from rain set no fruit; second, among the 14 sites we investigated fruit-set in rain-protected plants was very low (approx. 3 %), an unexpected result if the species were capable of regular autonomous self-pollination; lastly, at the same sites only about 23 % of open-pollinated flowers produced fruit, a value much lower than is commonly found in orchid species capable of autonomous self-pollination (Tremblay *et al.*, 2005). These results, in concert with our observations and video recording of the details of the pollination process, provide strong evidence that rain plays the primary role in causing self-pollination in *A. rigida.* Unlike other species in which rain-assisted self-pollination appears to occur, *A. rigida* possesses a suite of floral adaptations that facilitate this process. These include vertically oriented cup-shaped flowers, which are absent from the remaining Chinese species of *Acampe*, a weak anther cap that is easily dislodged by raindrops causing pollinia to bounce upwards, and the intricate stretchable stipe that pulls the pollinia back in a precise way into the stigmatic cavity causing self-pollination.

Function of ombrophily

Autonomous self-pollination is most often interpreted as a mechanism of reproductive assurance when pollination conditions are uncertain (Lloyd, 1992; Eckert *et al.*, 2006). Autogamy in orchids is generally facilitated by the close proximity of anthers and stigmas and a diversity of specific floral mechanisms are reported (reviewed in Catling, 1990). Most involve modification of the pollinarium and/or the rostellum, allowing pollinia to directly contact the stigma of the same flower (Catling, 1990; Johnson and Edwards, 2000), although more complicated mechanisms are also known (Liu *et al.*, 2006; Micheneau *et al.*, 2008). Because autonomous selfing appears to be easily achieved in Orchidaceae, occurring in virtually every tribe and subtribe and 5–20 % of species (Catling, 1990), it is an interesting question as to why *A. rigida* has evolved an alternative strategy for self-pollination involving ombrophily.

In our study area the flowering period of *A. rigida* occurs during the wet season. Although rainless days are present during this time, most flowers are subjected to at least one significant rainfall event and, more commonly, they experience days of continuous rain. Under these environmental conditions, as our observations indicate, visits by pollinators are very infrequent, a problem that is no doubt exacerbated by the fact that the species is rewardless. By possessing the potential for outcrossing during rainless days, but employing rain-mediated self-pollination during wet weather, *A. rigida* may benefit from maintaining mating system flexibility. This option is often foreclosed in species with well-developed means of autonomous self-pollination. Floral mechanisms providing guaranteed autonomous self-pollination generally increase fruit-set; however, they also tend to promote high selfing rates, thus reducing opportunities for the production of high-quality progeny from outcrossing. Ombrophily in *A. rigida* may therefore represent an evolutionary compromise to these conflicting demands in an environmental context that limits the effectiveness of deceit pollination because of very low pollinator activity. However, this hypothesis depends on the timing of rainfall in relation to the anthesis period of flowers and thus the mode of self-pollination (Lloyd, 1979). If ombrophily is triggered by rain at the beginning of anthesis, opportunities for outcrossing are prevented, a situation equivalent to prior self-pollination. In contrast, if flowers initially experience fine weather but are not visited by pollinators, ombrophily can function in an equivalent manner to delayed self-pollination, guaranteeing reproductive assurance. Future studies could determine which of these situations most commonly occurs.

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