Water mediates fertilization in a terrestrial flowering plant

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Summary

- Water-mediated fertilization is ubiquitous in early land plants. This ancestral mode of fertilization has, however, generally been considered to have been lost during the evolutionary history of terrestrial flowering plants.
- We investigated reproductive mechanisms in the subtropical ginger *Cautleya gracilis* (Zingiberaceae), which has two pollen conditions – granular and filiform masses – depending on external conditions. We tested whether rain transformed granular pollen into filiform masses and whether this then promoted pollen-tube growth and fertilization of ovules. Using experimental manipulations in the field we investigated the contribution of water-mediated fertilization to seed production.
- Rain caused granular pollen to form filiform masses of germinating pollen tubes, which transported sperm to ovules, resulting in fertilization and seed set. Flowers exposed to rain produced significantly more seeds than those protected from the rain, which retained granular pollen. Insect pollination made only a limited contribution to seed set because rainy conditions limited pollinator service.
- Our results reveal a previously undescribed fertilization mechanism in flowering plants involving water-mediated fertilization stimulated by rain. Water-mediated fertilization is likely to be adaptive in the subtropical monsoon environments in which *C. gracilis* occurs by ensuring reproductive assurance when persistent rain prevents insect-mediated pollination.

Introduction

Green algae are the distant ancestors to terrestrial plants, and fertilization in this group occurs in aquatic environments mediated by water (Bold & Wynne, 1985; Clifton & Clifton, 1999). In early land plants, such as ferns, lycopods, horsetails and bryophytes (mosses, liverworts and hornworts), fertilization depends on a continuous layer of moisture, making these taxa poorly adapted to terrestrial conditions that experience extended dry periods (Longton & Schuster, 1983; Glime, 2013). Thus, water-mediated fertilization has been considered a unique feature of early land plants in which motile sperm are the agents of male gamete dispersal (Niklas, 1997; Raven *et al.*, 2005). By contrast, in seed plants (gymnosperms and angiosperms), this dependency on water is highly restricted because pollen, the highly reduced male gametophyte generation, is the agent of male gamete dispersal and is more resistant to desiccation than sperm (Pacini, 2000). In most seed plants fertilization is achieved following pollen dispersal by wind or animals (Faegri & Van der Pijl, 1971), although in the *c.* 350 species of hydrophilous angiosperms (0.1% of the angiosperms), water is

the primary agent of pollen dispersal (Sculthorpe, 1967; Cox, 1988; Ackerman, 2000).

Currently, the prevailing view is that water-mediated fertilization has been lost during the evolutionary history of terrestrial flowering plants because it is nonadaptive (Cronberg *et al.*, 2006). Indeed, fertilization mechanisms independent of water represent a significant reproductive innovation that is intimately associated with the ecological and evolutionary success of seed plants. Nevertheless, flowers have numerous opportunities to encounter water, especially in the form of rain, raising the possibility that water-mediated fertilization may have been overlooked in some terrestrial angiosperms and could have adaptive value in environments that experience persistent rain.

Flowering plants release pollen to the external environment during anthesis. Pollen is dehydrated and quiescent at this stage, protected and encased in a relatively impermeable hard sporopollenin coat maintaining viability during dispersal. Pollen grains subsequently rehydrate and germinate after landing on stigmatic surfaces, some of which are wet (reviewed in Taylor & Hepler, 1997; Franklin-Tong, 2010). It is usually fatal for pollen to contact water before adhering to the stigma, and diverse morphological and

biochemical mechanisms have evolved to protect pollen from rain (Eisikowitch & Woodell, 1974; Mao & Huang, 2009). Pollen grains can burst in less than a few minutes in numerous species (Corbet & Plumridge, 1985; Mao & Huang, 2009), and indeed water is used as an emasculation tool for the production of hybrid cotton (*Gossypium hirsutum*), because cotton pollen loses viability after a short exposure to water (Burke, 2002). Water has been a significant abiotic selective force in the reproduction biology of flowering plants (Jones, 1967; Sun *et al.*, 2008; Mao & Huang, 2009; Wang *et al.*, 2010), with most research focused on strategies that ameliorate the harmful effects of water on pollination and mating. By contrast, the potentially positive effects of water on plant reproduction in terrestrial angiosperms have been largely ignored, aside from the obvious requirements of water for plant growth.

Here, we report a previously unknown water-mediated fertilization mechanism in a terrestrial flowering plant. *Cautleya gracilis* (Smith) Dandy (Fig. 1a), a perennial herb in the ginger family (Zingiberaceae), occurs in moist valleys above 1800 m asl in subtropical southwestern China. Flowering occurs from June to August during the monsoon season. Individual flowers open in the early morning and wither during late evening. Despite their brief period of anthesis, most flowers experience rain owing to the season in which they flower. In our preliminary observations of stamens in the field, we discovered two pollen conditions: granular (Fig. 1b) and a filiform mass (Fig. 1c) depending on external weather conditions. This discovery motivated us to evaluate the hypothesis that pollen transformation was induced by water (rain) and played a key role in reproduction.

We begin by documenting the floral biology of *C. gracilis*, including pollinator observations, compatibility status and the facility for autonomous self-pollination and the timing of this process. Having established this basic information, we then addressed three questions: under field conditions does rain stimulate pollen germination and pollen-tube growth; what is the contribution of water-mediated fertilization to open-pollinated seed production on rainy days; to what extent does pollinator-mediated pollination contribute to seed production? Our results revealed three distinct reproductive mechanisms causing seed set in *C. gracilis*: pollinator-mediated pollination and autonomous self-pollination during rainless days, and rain-mediated self-fertilization on rainy days. Rain caused pollen to germinate and develop pollen tubes transporting sperm to ovules and achieving fertilization. This novel fertilization strategy appears to be the primary cause of seed production in the species.

Materials and Methods

Study system and sites

Our research on *C. gracilis* was conducted during five flowering seasons from 2009 to 2013. We selected populations at three sites to conduct field studies in Yunnan province, southwestern China: Lincang (LC population, 24°10′N, 99°37′E, altitude 2260 m), Yangbi (YB population, 25°40′N, 100°02′E, altitude 2340 m) and Ailao Mountain (AM population, 24°32′N, 101°01′E, altitude 2480 m). The distance between each population is *c.* 100 km. The

majority of the fieldwork was conducted at LC. *C. gracilis* inhabits rock walls in moist valleys at LC and YB, but is epiphytic on trees at AM. Individuals of *C. gracilis* can produce several inflorescences, with only one flower open at a time on each inflorescence.

Floral biology, pollinator observations and compatibility status

To collect basic information on the floral biology of *C. gracilis*, we randomly selected *c.* 30 plants at LC and recorded the number of flowers per inflorescence, the timing of anthesis, floral longevity, pollen condition and we also determined the autonomous self-pollination mechanism. We chose *c.* 20 flowering individuals to record observations of insects visiting flowers and their foraging behavior from early morning to late evening for 6 d in each population, in both rainy and rainless conditions during each July from 2009 to 2013.

To determine the compatibility status and facility for autonomous self-pollination, we randomly selected single flowers from *c.* 90 plants scattered throughout the population at LC during July 2010, and these were bagged before anthesis and evenly assigned to three treatments during rainless days: flowers hand-pollinated using pollen grains from the same flower and bagged (self-pollination); flowers emasculated before anthesis, hand-pollinated during anthesis with pollen from other individuals and bagged (cross-pollination); and flowers unmanipulated and bagged to prevent insect visitors (autonomous self-pollination). We collected mature fruits 30 d after the treatments to determine seed number. We used a generalized linear model to test for differences in seed number among the three treatments, followed by a least significant difference (LSD) test for multiple comparisons. Finally, we also emasculated and bagged *c.* 30 flowers before anthesis to test for apomixis, but we found no evidence for this phenomenon in the species.

When does autonomous self-pollination occur during rainless days?

To determine the timing of autonomous self-pollination in *C. gracilis*, we selected single flower buds from *c.* 210 plants at LC, and these were bagged to prevent insect visitors during the entire 1 d period of anthesis. We randomly and evenly assigned flowers among seven groups: anthers removed 2.5 h after anthesis; anthers removed 4 h after anthesis; anthers removed 6 h after anthesis; anthers removed 8 h after anthesis; anthers removed 10 h after anthesis; anthers removed 12 h after anthesis; and anthers left intact (NC). We collected mature fruits 30 d after the treatments to determine seed number. We used a generalized linear model to examine differences in seed number followed by an LSD test for multiple comparisons.

Does rain-induced germination of pollen result in ovule fertilization?

First, we examined the different pollen conditions in *C. gracilis* to determine whether the filiform pollen comprised germinated
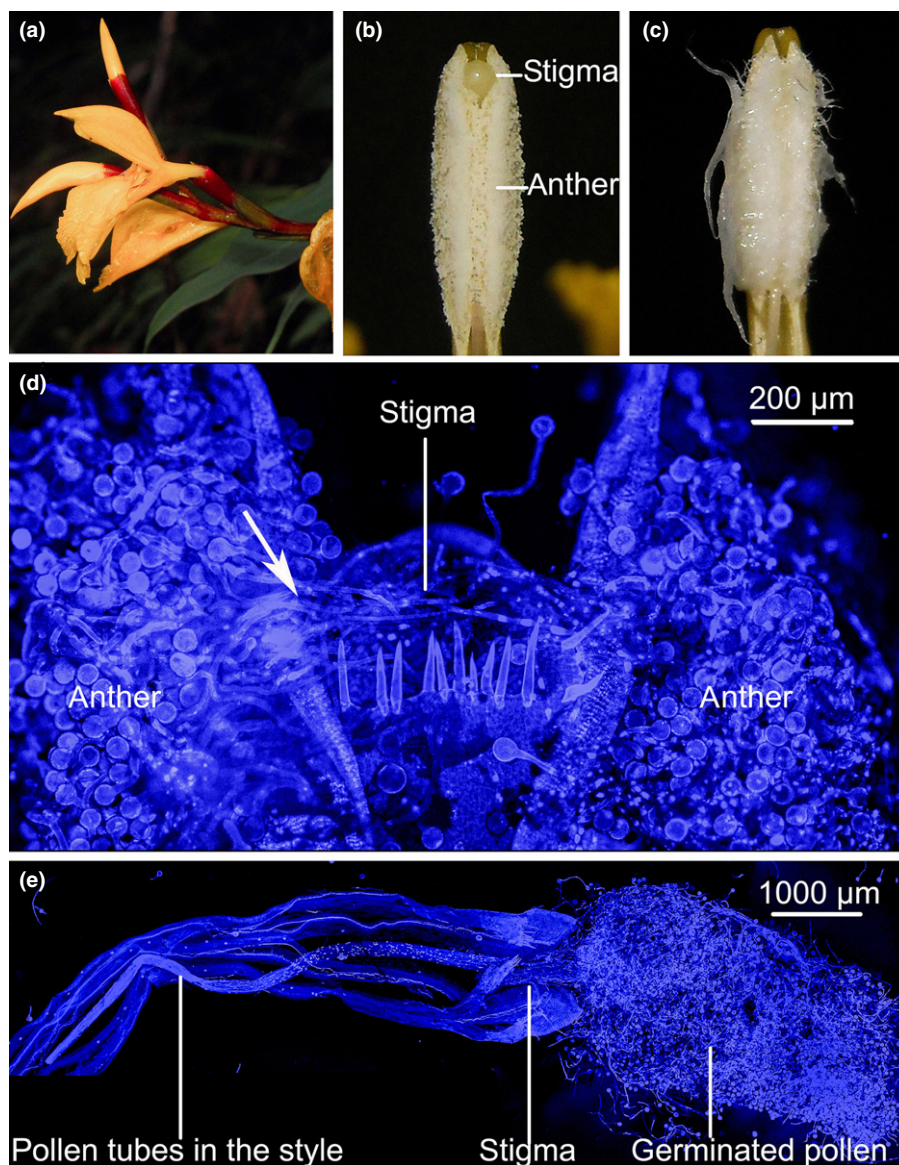
Fig. 1 Water-mediated fertilization in *Cautleya gracilis*. (a) Flower of *C. gracilis*; (b) anther in dry condition illustrating granular pollen on the anther and closely associated stigma; (c) anther and stigma from a flower subjected to rain showing mass filiform pollen tubes; (d) micrograph from a flower subjected to rain; the arrow identifies pollen tubes elongated from a naturally wetted anther towards the stigma; (e) panoramic micrograph from a flower subjected to rain, showing numerous pollen tubes that have grown from the anther through the stigma into the style. For (d) and (e), the tissue was stained with aniline blue and observed under a fluorescence microscope.

pollen with pollen tubes. To confirm this, we fixed samples of anthers with the granular and the filiform pollen in FAA solution (formaldehyde, acetic acid, ethanol at 1 : 1 : 18 ratio) in populations at all three research sites. Each sample was later stained with aniline blue (0.1% in 0.1 mol l^{-1} CH$_3$COOK) and observed under a fluorescence microscope (Zeiss LSM710; Carl Zeiss AG, Oberkochen, Germany).

We then hypothesized that the *in situ* pollen germination in the field was caused by rain. To test this, we performed two separate experiments. In the first experiment, single flowers from *c.* 150 individuals were randomly selected on rainy days in LC during July 2011. We bagged inflorescences with single flowers using nylon mesh, which excluded visitors but allowed rain to permeate bags. Plants were evenly and randomly allocated to two treatments: bagged flowers were protected from rain using plastic covers; and bagged flowers were left intact and wetted by rain. We then determined the condition of pollen grains at 2 h after the treatments were set up. In the second experiment, to further

investigate the effect of water on pollen germination, we collected granular pollen grains from single flowers from 20 plants on a sunny morning in LC. We then performed two treatments under field conditions: in the first, *c.* 50% of the pollen grains from each flower were placed on a strip of dry filter paper on a glass slide; and in the second, the remaining pollen grains were placed on moist filter paper on a glass slide. We then examined the condition of pollen grains 2 h after the treatments under a stereo microscope (Leica M50; Leica Camera AG, Wetzlar, Germany). The filter paper with germinated pollen was fixed in FAA solution for further laboratory observation.

In addition, we determined if rain-induced pollen germination results in ovule fertilization by tracking the growth of pollen tubes in pistils. Single flowers from 30 individuals were randomly selected on days with early morning rain in LC during July 2011 and 2012. We bagged inflorescences using nylon mesh before flowering, which excluded visitors but allowed rain to permeate bags. We collected the pistils and anthers with *in situ* germinated

pollen at 08:00 h when autonomous self-pollination had not occurred, thus excluding pollen from autonomous self-pollination. We fixed the samples of anthers and pistils in FAA solution. Each sample was later stained with aniline blue and the growth of pollen tubes and ovule penetration was observed under a fluorescence microscope.

What is the contribution of water-mediated fertilization to seed production?

We hypothesized that rain-mediated ovule fertilization would promote seed production of *C. gracilis* during rainy conditions. To test this hypothesis, we compared the seed set of bagged flowers either exposed to or sheltered from the rain in the LC population during the monsoon season. In addition, we evaluated whether delayed self-pollination might also promote seed production in *C. gracilis*. To determine this, we compared the seed set of bagged flowers either with anthers removed at 16:00 h or left intact, on both rainy and rainless days. We selected two patches (A and B) separated by 1.5 km to conduct the experiments at LC in July 2011. In each patch, we randomly selected single flowers from *c.* 320 plants and visitors were excluded during the entire 1 d period of anthesis. We evenly divided flowers from each patch into two groups: plants sheltered by plastic covers during the entire 1 d period of anthesis to protect pollen from daily rain and thus pollen germination on anthers (group 1); and plants exposed to rain (group 2). We checked each flower at 16:00 h on the day of anthesis to confirm that pollen from group 1 was ungerminated and pollen from group 2 was germinated on anthers. We collected mature fruits 30 d after the treatments to determine seed number. Note that a preliminary experiment demonstrated that sheltering on rainy days did not affect seed set. We used a generalized linear fixed effects model to test for differences in seed number per fruit among treatments because of the nonnormality of data, with pollen condition, treatment, patch and their interaction considered in the analysis.

We predicted that rain-mediated fertilization would maximize ovule fertilization under rainy conditions. To test this, we compared seed production from rain-mediated fertilization with what occurs when flowers are hand self-pollinated. We randomly selected single flowers from *c.* 100 plants at LC and performed two treatments in July 2011: in the first, *c.* 50 flowers were sheltered by plastic, bagged and hand self-pollinated on rainy days; in the second, *c.* 50 flowers were bagged and exposed to rain on rainy days. We collected fruits *c.* 30 d after treatments for seed counting. We compared seed number per fruit between the two treatments using a *t*-test.

To further evaluate the importance of rain-mediated fertilization to seed production, we determined the contribution of insect pollinators to seed production. This was assessed by quantifying the contribution of seed production by pollinator-mediated pollination under both rainless and rainy conditions at LC and YB in 2012. We evenly assigned single flowers from *c.* 120 plants to two groups at each site: in one group, plants were exposed to rainless days, and in the other, plants were exposed to rainy days. For each group, flowers were evenly allocated to two treatments:

open-pollination (i.e. intact and unmanipulated) seed set, a treatment that provides information on open-pollinated seed production; and emasculation of flowers before anther dehiscence and exposure to pollinators. The second treatment was designed to prevent intrafloral self-fertilization, but allowed pollination by pollinators. Therefore, the seed production for this treatment indicates the potential for animal pollination and the availability of pollinators under different weather conditions. We collected fruits *c.* 30 d after treatments for seed counting. We used a generalized linear fixed effects model to test for differences in seed number per fruit among treatments because of the nonnormality of data, with anther removal, environment, population and their interaction considered in the analysis.

To evaluate whether rain had an effect on seed production apart from facilitating pollen germination, we randomly selected single flowers from *c.* 100 plants and performed two treatments during July 2011 in LC: in the first, *c.* 50 flowers were bagged and hand self-pollinated on sunny days; and in the second, *c.* 50 flowers were sheltered, bagged and hand self-pollinated on rainy days. We quantified seed set per fruit and used a *t*-test to determine if there was a difference in seed number between the two treatments.

In our study, all analyses were performed using the statistical software R v.3.3.0 (R Core Team, 2016). Data for all *t*-tests were checked for normality and homogeneity of variance and were transformed as needed. All the generalized linear models were run using the CAR package with the quasi-Poisson distribution because of the overdispersed data (Fox *et al.*, 2013).

Results

Floral biology, pollinator observations and compatibility status

Cautleya gracilis had 6.9 ± 0.3 flowers (mean $\pm$ SE) per inflorescence, with one flower open at a time on each inflorescence. Flowers were scented and opened synchronously at *c.* 04:00 h in each population and withered at *c.* 21:00 h, lasting < 1 d. Under rainless conditions, *C. gracilis* pollen was granular (Fig. 1b; Supporting Information Fig. S1a). In the LC population, two species of bees (*Elaphropoda magrettii* and *Amegilla yunnanensis*) were the main pollinators of flowers contacting sex organs during foraging for nectar. On rainless days we observed a mean of 2.8 ± 0.31 visits h^{-1} ($n = 25$ h of observation). By contrast, on rainy days during the monsoon period, visitation by pollinators was much reduced, 0.08 ± 0.05 visits h^{-1} over 33 h of observation. In populations YB and AL, we observed no insect visitors, regardless of whether or not it rained.

We found that stigmatic fluid was continuously secreted during the flowering of *C. gracilis* and this formed a globule on the stigma at *c.* 10:00 h (Fig. 1b). Pollen grains close to the stigma adhered to the stigmatic fluid, thus achieving autonomous self-pollination on rainless days. This autonomous self-pollination mechanism has previously been reported in another species of ginger (*Roscoea debilis*; Fan & Li, 2012), which is the sister genus of *Cautleya*.

There was no significant difference in seed number per fruit between hand self- or cross-pollinated flowers (Fig. 2), indicating

that plants of *C. gracilis* are highly self-compatible. Bagged flowers produced significantly lower seed set than hand-pollinated flowers during rainless days (Fig. 2), indicating that, although *C. gracilis* has some capacity for autonomous self-pollination during rainless days, it is not sufficient to provide full reproductive assurance under pollen-limited conditions.

When does autonomous self-pollination occur during rainless days?

A generalized linear model indicated that mean seed number per fruit resulting from autonomous self-pollination differed significantly among the treatments in which anthers were removed at different times of the day in bagged flowers. Mean seed production per flower increased steeply between 4 and 6 h after the beginning of anthesis, but from 10:00 h onwards, seed set remained the same (Fig. 3). This indicates that most autonomous self-pollination of flowers occurs at *c.* 10:00 h (6 h after anthesis) once stigmatic fluid has accumulated and pollen grains have adhered to stigmas.

Does rain-induced germination of pollen result in ovule fertilization?

In contrast to our observations in the LC population on rainless days, discussed earlier, pollinators of *C. gracilis* were very scarce during persistent rain typical of monsoon conditions. During rain, petals failed to protect anthers from rain and, as a result, granular pollen on anthers became wet and was transformed into a filiform mass (Fig. 1c). Fluorescence microscopy confirmed that the granular pollen condition involved ungerminated pollen grains (Fig. S1a), whereas the filiform mass was composed of numerous germinated pollen grains with pollen tubes (Fig. S1b).

Our results revealed that on rainless days (70 flowers), on dry filter paper (20 flowers), or in flowers experimentally sheltered from rain (74 flowers), pollen remained granular and ungerminated (Figs 1b, S1a). By contrast, pollen germinated and became

filiform when placed on wet filter paper (20 flowers) (Fig. S1c), or when flowers were exposed to rain (70 flowers) (Figs 1c, S1b). In the moist filter paper treatment, pollen tubes were intertwined together in a mass and it was therefore impossible to quantify the number of germinating pollen grains (Fig. S1c). Fluorescence microscopy confirmed that pollen germinated when flowers were exposed to rain in all three *C. gracilis* populations. We therefore demonstrated that the process of wetting triggered pollen germination, pollen-tube growth and the development of a filiform mass on anthers.

By tracking pollen tubes on anthers with germinated pollen, we observed that following germination, pollen tubes grew from the anther to the stigma and traversed the style into the ovules and achieved fertilization (Fig. 1d,e). We therefore demonstrated that water-mediated pollen germination can result in ovule fertilization.

What is the contribution of water-mediated fertilization to seed production?

We found that flowers exposed to rain produced significantly more seeds than those protected from rain (Fig. 4a; Table 1), demonstrating that rain-mediated ovule fertilization promotes seed production of *C. gracilis* during rainy conditions. In addition, we compared the seed set of bagged flowers either emasculated at 16:00 h or left intact, under both rainy and rainless conditions and found no significant difference in seed set between the treatments (Fig. 4a; Table 1). This result indicates that delayed self-pollination mechanisms influencing seed set do not occur in either rainy or rainless conditions and further supports our contention that water-mediated fertilization is the primary source of seed production when pollinators are absent. Significantly, flowers experiencing rain-mediated fertilization produced as many seeds as hand self-pollinated flowers (*t*-test, df = 75, *t* = 1.05, *P* = 0.296), indicating that pollen wetting maximizes ovule fertilization under rainy conditions.
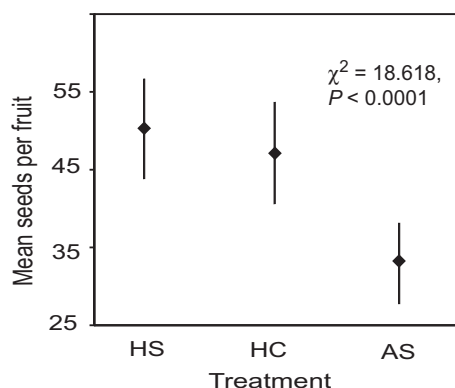

Fig. 2 Compatibility status and capacity for autonomous self-pollination in *Cautleya gracilis*. Flowers were bagged during the entire anthesis period and the three experimental treatments were hand self-pollination (HS), hand cross-pollination (HC) and intact unmanipulated (AS), with a total of 154 plants used. Values plotted are observed means and 95% confidence intervals. Chi-squared (χ^2) and *P*-values are from likelihood ratio tests.
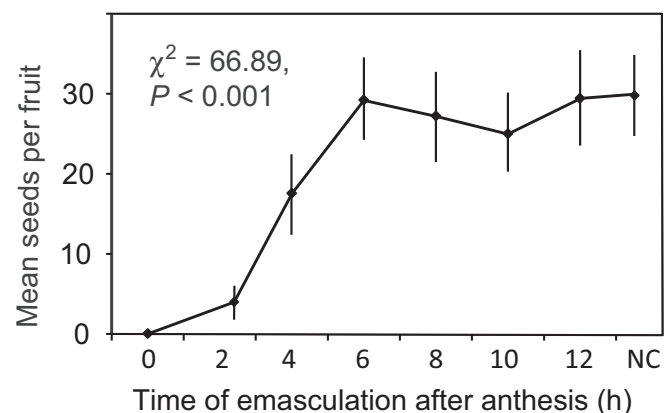

Fig. 3 Relationship between the time of emasculation and seed production by autonomous self-pollination in *Cautleya gracilis*. Flowers were bagged during the entire anthesis period and flowers were emasculated at different times after the start of anthesis, with a total of 274 plants used. NC, bagged and unmanipulated flowers. Values plotted are observed means and 95% confidence intervals. Chi-squared (χ^2) and *P*-values are from likelihood ratio tests.

Exposed flowers from which anthers had been produced significantly fewer seeds than intact flowers under open pollination during both rainless and rainy conditions (Fig. 4b; Table 2). In LC, pollinator-mediated pollination during rainless periods occurred, but made only a limited contribution to seed set in comparison with intact flowers. In YB, there was no evidence of pollinator-mediated pollination, regardless of whether or not it rained, probably because no pollinators were observed at this site (Fig. 4b; Table 2). Collectively these results indicate that pollinator-mediated pollination makes a limited contribution to seed production.
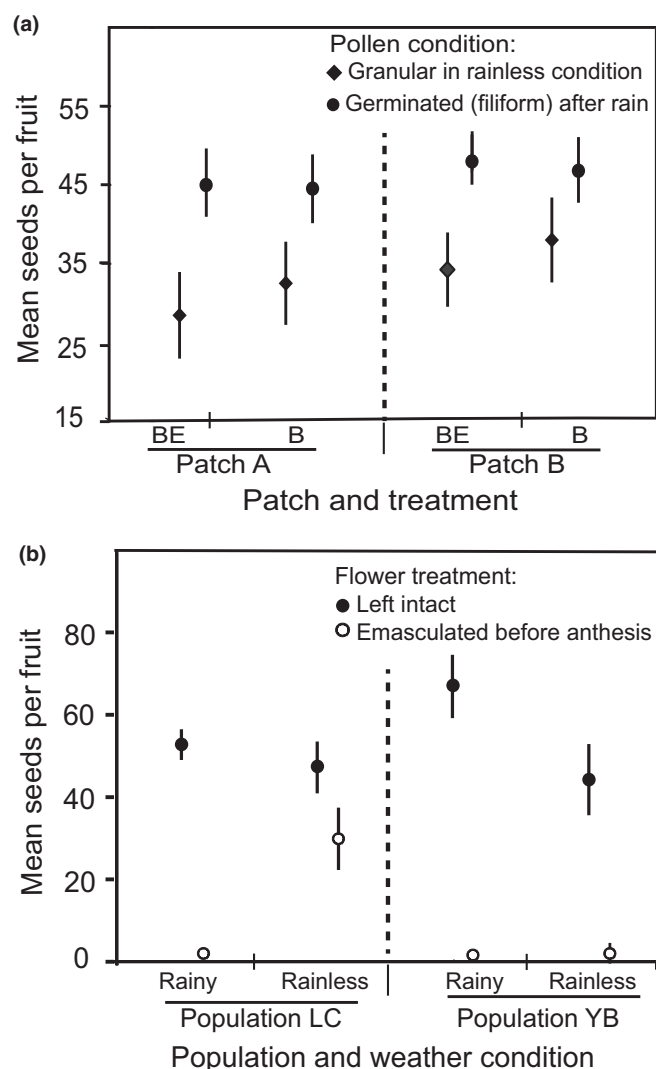

Fig. 4 Variation in seed production in experimental pollination treatments of *Cautleya gracilis*. (a) Contribution of rain-mediated fertilization to seed production in the Lincang population (LC). Flowers with germinated pollen on the anther (plants exposed to rain) had significantly higher seed production than flowers with granular pollen (plants sheltered from rain), with a total of 522 plants used in experiments. Treatments: BE, flowers bagged during the entire flowering period and emasculated at 16:00 h; B, flowers bagged during the entire flowering period. (b) Contribution of pollinator-mediated pollination to seed production in LC and Yangbi (YB), with a total of 303 plants used in experiments. Values plotted are observed means and 95% confidence intervals.

Table 1 Analysis of deviance with a generalized linear fixed effects model examining effects of pollen condition (granular in rainless condition vs germinated after rain), treatment (bagged vs bagged and emasculation at 16:00 h), patch (A vs B) and their interaction on seed set in *Cautleya gracilis*.

Variables	df	χ^2	P
Pollen condition (PC)	1	56.907	**< 0.0001**
Treatment	1	1.273	0.2593
Patch	1	7.49	**0.0062**
PC × treatment	1	2.82	0.0931
PC × patch	1	2.525	0.1121
Treatment × patch	1	0.284	0.5941
PC × treatment × patch	1	0.164	0.6858

Values of chi-squared (χ^2) and P are from likelihood ratio tests, and values in bold type indicate statistically significant effects.

Finally, there was no difference in seed production between flowers that were bagged and hand self-pollinated on sunny vs rainy days (*t*-test, *t* = −1.1998, df = 80, *P* = 0.23), indicating that rain has no effect on seed production apart from facilitating pollen germination.

Discussion

We have shown that rainwater causes pollen germination in *C. gracilis* and that pollen tubes transport sperm to ovules, resulting in fertilization and seed production. To our knowledge, this is the first demonstration of water-mediated fertilization under natural conditions in a terrestrial flowering plant. For a plant species such as *C. gracilis*, which flowers during the monsoon period when daily rainfall is common, the occurrence of water-mediated fertilization is consistent with the hypothesis that this reproductive mechanism is of adaptive value maintaining fertility at a time of the year when pollinator service is limited.

Unlike early land plants that possess motile sperm (e.g. bryophytes, lycophytes and ferns), angiosperms rely on pollen tubes to deliver sperm to ovules. Despite this difference, water-mediated fertilization in *C. gracilis* is similar to these groups in

Table 2 Analysis of deviance with a generalized linear fixed effects model examining effects of anther removal (flower intact vs anthers removed before anthesis), environment (rainy vs rainless), population (LC vs YB) and their interaction on seed set in *Cautleya gracilis*.

Variables	df	χ^2	P
Anther removal (AR)	1	440.79	**< 0.0001**
Environment	1	0.16	0.6932
Population	1	4.07	**0.0436**
AR × environment	1	92.02	**< 0.0001**
AR × population	1	80.49	**< 0.0001**
Environment × population	1	4.48	**0.0344**
AR × environment × population	1	0.7	0.4038

Values of chi-squared (χ^2) and P are from likelihood ratio tests, and values in bold typeface indicate statistically significant effects.

two important ways: water is necessary to achieve sperm release, at least when pollinators are absent; and only sperm is transported and released from the male gametophyte during fertilization. These features of *C. gracilis* distinguish it from documented examples of rain-mediated pollination in other angiosperm species. For example, in the orchid *Acampe rigida*, which also flowers during the monsoon season in subtropical southwestern China, the entire pollinia are physically ejected from anthers and transported to stigmas by raindrops, without sperm release (Fan *et al.*, 2012). Water-mediated pollen germination is also recorded in *Primula* (Eisikowitch & Woodell, 1974) and *Arabidopsis* mutants (Johnson & McCormick, 2001; Coimbra *et al.*, 2010; Xie *et al.*, 2010; Ju *et al.*, 2016), in which pollen grains germinate after immersion in water for several hours or under high humidity conditions. However, these cases are not associated with fertilization and, at least in wild species, are probably maladaptive because they result in male gamete wastage. Pollen germination and fertilization within floral buds occur in numerous cleistogamous species (Anderson, 1980; Mayers & Lord, 1983; Culley & Klooster, 2007), but this generally results from precocious development of pollen in cleistogamous flowers without a dehydration period and rain stimulus (Mayers & Lord, 1983; Taylor & Hepler, 1997; Culley & Klooster, 2007).

We propose that the occurrence of water-mediated fertilization in *C. gracilis* is probably an adaptive reproductive mechanism, given the particular climatic context in which this subtropical ginger occurs. Rain generally reduces pollinator activity and wetting further reduces pollen viability for most angiosperms (Mao & Huang, 2009). Rainy conditions are therefore usually detrimental to both pollinator-mediated pollination and autonomous self-pollination. Flowering of *C. gracilis* occurs from June to August when there is an average of 22 d month^{-1} of rain and 50% of the annual precipitation falls, based on our analysis of 30 yr climate data for the regions where our study populations occur (Table S1). For example, rain was intense and frequent in the LC population, falling, on average, on 89 of the 103 observation days during peak flowering from 2010 to 2012. Under these inclement environmental conditions our observations of pollinator activity indicated that bee visitation to *C. gracilis* flowers was severely curtailed. Under these conditions, floral mechanisms promoting reproductive assurance through self-fertilization seem likely to be adaptive, as also hypothesized for *A. rigida* (Fan *et al.*, 2012). Rain-mediated fertilization during the monsoon season is therefore more likely to be a dominant reproductive mode than simply a complement to insect pollination (Fig 4b).

Under rainless conditions, pollen of *C. gracilis* is granular and pollinators mediate pollen dispersal among plants, as observed in LC. However, even when pollinators are not available on rainless days, such as occurred in YB and AM, autonomous self-pollination occurs 4–6 h after the beginning of anthesis. This mode of autonomous self-pollination enables some seed production, although significantly less than under rainy conditions (Fig. 4a). Future experimental work on *C. gracilis* would be valuable to rigorously test the reproductive assurance hypothesis. In particular, it will be important to determine the contribution of outcrossing and the various modes of selfing to female and male fitness using floral manipulations and genetic markers (see Schoen & Lloyd, 1992; Eckert *et al.*, 2006; Busch & Delph, 2012).

Pollinators were not observed in two of the three populations of *C. gracilis* that we investigated in this study and thus rain-mediated fertilization is likely to have been the primary mechanism causing seed set (Fig. 4b). We do not know the specific cause(s) of the apparent absence of pollinator service at AM and YB. It is possible that our relatively limited observations failed to detect low activity levels at these sites. The environmental conditions of *C. gracilis* habitats and persistent rain during the flowering season would probably induce chronic pollinator limitation because rainy conditions generally limit bee activity (Kleinert-Giovannini & Imperatriz-Fonseca, 1986) and promote floral mechanisms providing reproductive assurance. In addition, population size and density in flowering plants are important determinants of pollinator abundance (Bernhardt *et al.*, 2008). Populations of *C. gracilis* at AM and YB were much smaller and occurred at a lower density than at LC, which comprised *c.* 3000 individuals. At AM there were *c.* 500 individuals distributed as epiphytes on numerous trees with several individuals on each tree, and at YB there were *c.* 900 individuals. Thus, populations at YB and AM may have been sufficiently unattractive to pollinators compared with LC, potentially accounting for our failure to observe any visitation by pollinators to flowers at these sites.

Water-mediated fertilization in early land plants originated from algal ancestors. Such an origin is unlikely in *C. gracilis* because, in common with all angiosperms, this species does not possess motile sperm. It is more probable that this fertilization strategy has evolved *de novo* from animal pollination. Close relatives in the Zingiberales typically possess this widespread pollination system (Specht *et al.*, 2012) and *C. gracilis* exhibits many of the characteristic features of animal pollination, including moderately showy, scented flowers and nectar. Indeed, our field observations confirmed bee pollination. The relative importance of animal pollination vs rain-mediated fertilization in *C. gracilis* probably depends on the climatic context in which local populations occur and is likely to vary both spatially and temporally. Flexibility in reproductive function is a hallmark of angiosperms and this may enable *C. gracilis* to persist across a wide range of environments varying in rainfall intensity. Comparative analysis of additional angiosperm species, including other taxa of Zingiberales that primarily flower during the rainy season in other regions of the world, would be worthwhile to determine whether water-mediated fertilization is more widely distributed in flowering plants.

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Author contributions

Y-LF, SCHB, J-LZ, Y-MX and Q-JL designed the research; Y-LF, SCHB and Q-JL wrote the manuscript; Y-LF and J-QY performed experiments; and Y-LF and J-QY analyzed data and prepared the figures. All authors read, corrected and approved the manuscript.

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References

Ackerman JD. 2000. Abiotic pollen and pollination: ecological, functional and evolutionary perspectives. *Plant Systematics and Evolution* 222: 167–185.

Anderson W. 1980. Cryptic self-fertilization in the Malpighiaceae. *Science* 207: 892–893.

Bernhardt CE, Mitchell RJ, Michaels HJ. 2008. Effects of population size and density on pollinator visitation, pollinator behavior, and pollen tube abundance in *Lupinus perennis. International Journal of Plant Sciences* 169: 944–953.

Bold HC, Wynne MJ. 1985. *Introduction to the algae: structure and reproduction.* Englewood Cliffs, NJ, USA: Prentice Hall.

Burke JJ. 2002. Moisture sensitivity of cotton pollen: an emasculation tool for hybrid production. *Agronomy Journal* 94: 883–888.

Busch JW, Delph LF. 2012. The relative importance of reproductive assurance and automatic selection hypotheses for the evolution of self-fertilization. *Annals of Botany* 109: 553–562.

Clifton KE, Clifton LM. 1999. The phenology of sexual reproduction by green algae (Bryopsideales) on Caribbean coral reefs. *Journal of Phycology* 35: 24–34.

Coimbra S, Costa M, Mendes MA, Pereira AM, Pinto J, Pereira LG. 2010. Early germination of *Arabidopsis* pollen in a double null mutant for the arabinogalactan protein genes *AGP6* and *AGP11. Sexual Plant Reproduction* 23: 199–205.

Corbet SA, Plumridge JR. 1985. Hydrodynamics and the germination of oil-seed rape pollen. *Journal of Agricultural Science* 104: 445–451.

Cox PA. 1988. Hydrophilous pollination. *Annual Review of Ecology and Systematics* 19: 261–280.

Cronberg N, Natcheva R, Hedlund K. 2006. Microarthropods mediate sperm transfer in mosses. *Science* 313: 1255.

Culley TM, Klooster MR. 2007. The cleistogamous breeding system: a review of its frequency, evolution, and ecology in angiosperms. *Botanical Review* 73: 1–30.

Eckert CG, Samis KE, Dart S. 2006. Reproductive assurance and the evolution of uniparental reproduction in flowering plants. In: Harder LD, Barrett SCH, eds. *Ecology and evolution of flowers.* Oxford, UK: Oxford University Press, 183–203.

Eisikowitch D, Woodell SRJ. 1974. The effect of water on pollen germination in two species of *Primula. Evolution* 28: 692–694.

Faegri K, Van der Pijl L. 1971. *The principles of pollination ecology.* Oxford, UK: Pergamon Press.

Fan XL, Barrett SCH, Lin H, Chen LL, Zhou X, Gao JY. 2012. Rain pollination provides reproductive assurance in a deceptive orchid. *Annals of Botany* 110: 953–958.

Fan YL, Li QJ. 2012. Stigmatic fluid aids self-pollination in *Roscoea debilis* (Zingiberaceae): a new delayed selfing mechanism. *Annals of Botany* 110: 969–975.

Fox J, Weisberg S, Adler D, Bate D, Baud-Bovy G, Ellison S, Firth D, Friendly M, Gorjanc G, Graves S et al. 2013. *R package car: companion to applied regression. v.3.0-2.* [WWW document] URL http://CRAN.R-project.org/package=car [accessed 14 August 2018].

Franklin-Tong N. 2010. Plant fertilization: bursting pollen tubes! *Current Biology* 20: 681–683.

Glime JM. 2013. Bryophyta-Bryopsida. In: Glime JM, ed. *Bryophyte ecology.* Ebook sponsored by Michigan Technological University and the International Association of Bryologists, 1–23.

Johnson SA, McCormick S. 2001. Pollen germinates precociously in the anthers of *raring-to-go*, an *Arabidopsis* gametophytic mutant. *Plant Physiology* 126: 685–695.

Jones C. 1967. Some evolutionary aspects of a water stress on flowering in the tropics. *Turrialba* 17: 188–190.

Ju Y, Guo L, Cai Q, Ma F, Zhu QY, Zhang Q, Sodmergen S. 2016. *Arabidopsis* JINGUBANG is a negative regulator of pollen germination that prevents pollination in moist environments. *Plant Cell* 28: 2131–2146.

Kleinert-Giovannini A, Imperatriz-Fonseca VL. 1986. Flight activity and responses to climatic conditions of two subspecies of *Melipona marginata* Lepeletier (Apidae, Meliponinae). *Journal of Apicultural Research* 26: 3–8.

Longton RE, Schuster RM. 1983. Reproductive biology. In: Schuster RM, ed. *New manual of bryology.* Nichinan, Japan: Hattori Botanical Laboratory, 386–462.

Mao YY, Huang SQ. 2009. Pollen resistance to water in 80 angiosperm species: flower structures protect rain-susceptible pollen. *New Phytologist* 183: 892–899.

Mayers AM, Lord EM. 1983. Comparative flower development in the cleistogamous species *Viola odorata.* II. An organographic study. *American Journal of Botany* 70: 1556–1563.

Niklas KJ. 1997. *The evolutionary biology of plants.* Chicago, IL, USA: University of Chicago Press.

Pacini E. 2000. From anther and pollen ripening to pollen presentation. *Plant Systematics and Evolution* 222: 19–43.

R Core Team. 2016. *R: a language and environment for statistical computing.* Vienna, Austria: R foundation for statistical computing. [WWW document] URL https://www.R-project.org/ [accessed 3 May 2016].

Raven PH, Evert RF, Eichhorn SE. 2005. *Biology of plants.* New York, NY, USA: W.H. Freeman.

Schoen DJ, Lloyd DG. 1992. Self- and cross-fertilization in plants. III. Methods for studying modes and functional aspects of self-fertilization. *International Journal of Plant Sciences* 153: 381–393.

Sculthorpe CD. 1967. *The biology of aquatic vascular plants.* London, UK: Edward Arnold Ltd.

Specht CD, Yockteng R, Almeida AM, Kirchoff BK, Kress WJ. 2012. Homoplasy, pollination, and emerging complexity during the evolution of floral development in the tropical gingers (Zingiberales). *Botanical Review* 78: 440–462.

Sun JF, Gong YB, Renner SS, Huang SQ. 2008. Multifunctional bracts in the dove tree *Davidia involucrata* (Nyssaceae: Cornales): rain protection and pollinator attraction. *American Naturalist* 171: 119–124.

Taylor LP, Hepler PK. 1997. Pollen germination and tube growth. *Annual Review of Plant Physiology and Plant Molecular Biology* 48: 461–491.

Wang Y, Meng LL, Yang YP, Duan YW. 2010. Change in floral orientation in *Anisodus luridus* (Solanaceae) protects pollen grains and facilitates development of fertilized ovules. *American Journal of Botany* 97: 1618–1624.

Xie B, Wang XM, Hong ZL. 2010. Precocious pollen germination in *Arabidopsis* plants with altered callose deposition during microsporogenesis. *Planta* 231: 809–823.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Pollen performance of *Cautleya gracilis* under rainy, rainless and experimentally wet filter paper conditions as observed by fluorescence microscopy.

Table S1 Precipitation data from 30 yr climate data for the area encompassing the study sites of *Cautleya gracilis*.

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