

Protogynous, pollen limitation and low seed production reasoned for the dieback of *Spartina anglica* in coastal China

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Abstract

The exotic species *Spartina anglica* in coastal China has been experiencing dieback in the past decade, while the mechanism of dieback is still not clear. It is important to discover the intrinsic reasons from breeding system since the population almost lose the sexual reproduction ability. A series of experiments were carried out to investigate the breeding system of the species in both the field and greenhouse. The flower was protogynous. Since there were irregular pollen grains, the pollen viability was $28.2 \pm 1.9\%$ and $20.8 \pm 0.9\%$, respectively in two sites. In the field, pollen grains arrived at the neighbors at most 3 m away. Because irregular pollen tube, only about one or two pollen tubes entered the micropyle in cross-pollination flower and hardly did in self-pollination. The proportion of seed production per inflorescence was as low as 5.9% in nature coastal China. Thus, protogynous, poor pollen quality, abnormal pollen grains and pollen tube were the main causes of low seed production, which led to *S. anglica* population mainly rely on asexual reproduction for population regeneration and finally induce the dieback of the species in coastal China. While, transplanting in greenhouse did not help the species restore its sexual reproduction capacity.

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1. Introduction

Plant dieback is a general phenomenon occurring in some plants of different vegetations [1,2]. During dieback, the plants will experience symptoms such as loss of foliage and bud death [3], and the vegetation area would shrink because of severe plant damage [1,4]. Understanding the cause of plant dieback is a precursor to successful management of these deteriorating vegetations and to controlling invasions if the species is an exotic invader.

Various mechanisms have been proposed to explain the dieback of this plant species. The dieback in a salt marsh dominated by *Spartina alterniflora* was due to excessive submergence [4] and drought stress [1]. Patch dieback of

Colophospermum mopane was influenced by vegetation structure, soil surface conditions and soil chemistry [2,5]. Besides these causes, pathogens were implicated as main cause of several dieback events [3,6]. Other suggested causes for dieback are interspecific competition [7], insect outbreak [8], and air pollutants [9]. Thus far, the factors being considered for contributing to dieback are mainly environmental with little attention paid to the self-regeneration of the target populations through sexual reproduction during plant dieback.

In fact, the breeding system and seed abortion could provide a basic background regarding the rarity of some endangered plants [10,11]. The rare plants had special breeding systems, which could be reasoned for their rare occurrence in natural conditions [12].

In some flowering plants, only a few of the flowers and ovules were initiated to form fruits and seeds [13]. Abortion of flowers and fruits could occur at various stages of development. Factors affecting seed formation acted both before and after fertilization and could be determined by analyzing the

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characteristics of ovules in the fruits that attain maturity [14]. The important factors that acted in the pre-fertilization were the limitation of pollen grains [15], self-incompatibility and gametic sterility [16]. Pollen grain deposition patterns may vary greatly in natural populations, thus affecting the opportunity of pollen competition for ovules and pollen-tube growth [17–19]. Pollen competition could in turn affect seed production, fruit set, and progeny vigor [20]. The second important mechanism that induced low seed set was self-incompatibility [21]. The other factors inducing seed abortion were maternal genotypes [22], resource limitation [23], and inbreeding depression [24–27].

Spartina anglica C.E. Hubbard, an English cordgrass, is a perennial salt marsh grass that arose through the hybridization of female parent *S. alterniflora* and male *S. maritima* [28]. It was deliberately introduced to China from Europe in 1963, mainly for cattle fodder, habitat reclamation, and the stabilization of dikes and protection of sea walls [29]. *S. anglica* has gone through five stages in China: laboratory propagation and trial planting (1963–1966), slow spread (1966–1973), rapid spread (1973–1985), natural maintenance (1985–1995), and the dieback period (1995–) [30,31]. The dieback of Chinese *S. anglica* is apparent in its decreasing acreage from about 40,000 ha (40°53'N–21°30'N) in the 1990s to only 50 ha in the field today, and with the decline in its culm height, growth rate and virtual loss of sexual reproduction [31]. Furthermore, the standing biomass and net biomass production are 215.2 g DW m⁻² and 112.4 g DW m⁻² year⁻¹, which is notably lower than the production of *S. anglica* in England and in pre-1990's Chinese populations [31]. However, the mechanism of *S. anglica* dieback in coastal China is still unclear.

Thus, it is necessary to study the breeding system in order to uncover reason for the *S. anglica* dieback in Chinese coast. The purpose of this study is to (1) supply some of the missing information on the breeding system of the dieback species, (2) determine which factor or factors in the breeding system make the species produce so few seeds in natural populations; and (3) clear the correlation between the low seed production and the dieback of the species.

2. Materials and methods

2.1. Study sites

A site where *S. anglica* was naturally well established and evenly distributed was selected for the field investigation. The site was situated in one of the largest *S. anglica* vegetation patches (120°15' E and 33°42' N) and located at the coast of the Yellow Sea in China. This area experiences an oceanic climate with monsoons. The averages for sunlight time per year and solar radiation are 2199–2362 h and 116.2–121.0 kcal cm⁻², respectively. The mean annual precipitation is 980–1070 mm with 70% falling between May and July. The mean annual temperature is 15–15.6 °C, with the average being 27.4 °C and the maximum temperature, 39.0 °C, occurring in July.

For the greenhouse experiments, hundreds of ramets were transplanted from the site of the field experiment to a

greenhouse located in Nanjing University, China (32°10'37" N, 118°41'57" E) in early April 2005. The ramets were cultivated in plastic trays (40 cm long × 30 cm wide × 35 cm deep), which were filled with a mixture of equal parts sand and clay. At the time of the experiment, salinity was maintained at 15 g NaCl l⁻¹ and all tanks were immersed to a depth of 30 cm.

2.2. The species

S. anglica is a stout, rhizomatous salt marsh grass that spreads mainly by clonal growth, often forming extensive meadows [32]. The plant is 50–100 cm tall, with stems of 5 mm or more in diameter. However, its growth form varies among its habitats [32,33]. The flowers occur in numerous, erect, contracted panicles, and consist of closely overlapping spikelets in two rows on one side of the rachis [34]. The flowers are wind pollinated and can produce viable seeds through both self- and cross-pollination in Europe [34]. However, seed production has changed significantly over years, especially in China [30].

2.3. Flower phenology and production survey

The time of flowering shoot appearance and flowering was recorded. The number of spikes were counted in each inflorescence ($n = 10$). The number of spikelets borne on each spike was counted in randomly collected 10 spikes ($n = 10$). Meanwhile, the numbers and the lengths of stamen, pistil and glume were determined based on 10 flowerlets randomly collected from 10 inflorescences in field or greenhouse experiments.

2.4. Pollen production and structure measurement

To determine the number of pollen grains per anther, mature anthers were harvested from five unopened flowerlets from five inflorescences, one flowerlet from each inflorescence. Anthers ($n = 15$) before un-dehisced were restored in five pyxises filled with 1.5 ml 1N HCl solution for 30 min to dissolve the wall of anther. The pollen liquid of 1.5 ml was removed and 0.1 ml of liquid was taken to calculate the amount of pollen under the microscope. Then the amount of pollen per anther and flowerlet was estimated based on the pollen number in the 0.1 ml of liquid. The technique was modified based on previous researches [15,35].

The pollen–ovule ratio was determined through dividing the average number of pollen grains by the average number of ovules per flowerlet. The number of pollen grains was determined by the pollen production per anther. The number of ovules in each ovary was determined by adding pressure to the carpel to open each ovary and counting the ovules under an anatomy microscope. Since there was only one ovule for each flowerlet, the pollen product of per flowerlet was equal to the pollen–ovule per flowerlet ($n = 5$).

Pollen viability at the shedding stage was estimated using a microscope [15,36]. The pollens of ten inflorescences ($n = 10$) from field and greenhouse experiments were measured in July

and May, respectively, when the pollens began shedding. The pollens from each inflorescence ($n = 10$) were placed on one slide. Then pollen grains were stained using a solution of 0.5% TTC. The red pollen was viable, and the rest was not. Finally, the viable pollen ratio per inflorescence could be calculated.

The shape and regularity of the pollens were investigated with a scanning electronic microscope. The pollens were first fixed in 2% glutaraldehyde for 18 h at 4 °C. Fixed materials were dehydrated through ascending series of cold acetone (10–100%, for 10 min each), dried at the critical point, mounted on aluminum stubs and coated with gold. Thirty pollen grains from each inflorescence were observed using Phillips SEM 505 ($n = 4$).

2.5. Pollen dispersal

To test pollen dispersal by the wind, glass slides (7.5 cm in length and 2.5 cm in width) covered with white vaseline on the upper surface were exposed to air for 1 week between 20 July and 27 July 2006. The wind was blowing almost squarely in the south-east direction. We selected a *S. anglica* patch with several flowering plants as the central point. Along the wind direction (south-east), six slides were placed 0.5 m apart from the central point at 9 a.m. and left for about 3.5 h, while another 24 slides were placed along north-east, east, south and south-west directions. In total, five different patches (points) were used in the investigation. The slides were collected to estimate the amount of pollen on the slides under a microscope and the pollen number per unit were calculated. At the same time, wind speed was determined by DAT-300 anemoscope [37,38].

2.6. The effect of pollination type on pollen-tube growth in stigmas

To determine the growth of pollen tube after pollination, we conducted manual pollination experiments in the field and greenhouse in July and May 2006, respectively. A plastic cocktail stick was used to remove pollen from the anthers. Before anthers dehiscing, pollination was achieved by gently applying the pollen on the stick tip to the feather stamen.

Twenty plants for each pollination type were bagged before flowering to prevent natural wind pollination. We designed two types of pollination treatments: (1) self-pollination, in which pollen from either the same flower or the other flowers on the same inflorescence were applied once via the cocktail stick; (2) cross-pollination, in which the pollens from the other plants were applied once via the cocktail stick. We emasculated flowers prior to pollen dehiscence, after which the treatment spikes were then bagged again.

Five flowerlets from each treatment were collected every 15 min for 90 min after pollination, then fixed in a 3:1 ethanol:acetic acid solution for 24 h, and stored in a 70% ethanol solution for later observation. Before observation, the fixed flowerlets were softened for 24 h with 8N NaOH, rinsed with water, and stained overnight with 0.01% aniline blue dye in a 0.1 M K_2HPO_4 solution [27,39]. The stamens were observed with a fluorescence microscope equipped with UV epifluorescence. At the same time, the number of pollen on the stamens was determined. The experiments were done in the greenhouse and the field during May and July, respectively.

2.7. Seed production observation

To examine the effect that different pollen origins have on viable seed production, a manual/natural pollination field experiment was carried out in July 2006. Twenty plants for each pollination type were artificially pollinated. Three types of pollination treatments were included in the experiment: (1) natural pollination, (2) self-pollination, and (3) cross-pollination. In early October, the well-developed seeds of different treatments were collected. The number of seeds per inflorescences ($n = 10$) was examined in a laboratory, and the proportion of seed production was thus measured through a ratio of the number of seed spikelets (with caryopsis) to the total spikelets per inflorescence.

2.8. Data analyses

The effects of different experimental sites on the observed number of stamens and pistils, the number of spikes per

Table 1
One-way ANOVA of the effect of different sites on the floral morphology and pollen parameters

Variables	Treatments			d.f.	F	p
	Marsh field	Greenhouse				
The first time of flowering	June	May		–	–	–
The number of stamens per flowerlet	3	3		9	–	–
The number of pistils per flowerlet	2	2		9	–	–
The number of spike per inflorescence	2.2 ± 0.5	2.2 ± 0.6		9	2.400	0.139
The number of spikelets per spike	9.8 ± 0.77	10.3 ± 0.93		9	0.171	0.684
The length of glume (cm)	1.88 ± 0.05	1.78 ± 0.06		9	1.339	0.262
Pistil length (cm)	1.41 ± 0.31	1.19 ± 0.30		9	2.506	0.131
Stamen length (cm)	1.42 ± 0.34	1.16 ± 0.27		9	3.418	0.081
The number of pollen grains per anther	771 ± 70	509 ± 44		14	12.699	0.011*
P/O	2312 ± 209.8	1528 ± 131.8		4	12.699	0.011*
Pollen viability ratio per inflorescence	0.282 ± 0.019	0.208 ± 0.009		9	11.199	0.015*
Irregular pollen percentage per inflorescence	37.5 ± 3.1%	33.1 ± 6.9%		3	1.279	0.273

Mean ± S.D., * $p < 0.05$.

inflorescence, the number of spikelets per spike, the lengths of glumes, pistils and stamens, the number of pollen grains per anther, P/O, the pollen viability percentage per inflorescence and irregular pollen percentage per inflorescence were analyzed by one-way ANOVA at the 0.05 significance level. The effects of time and pollination treatments (self-pollination and cross-pollination) on the parameters (the amount of pollen grains on a stigma, the amount of germinated pollen grain, the amount of abnormal pollen grains and the percentage of abnormal pollen tube) were analyzed by two-way ANOVA, with time and manner of pollination as fixed effect-independent variables. The pollen density distribution along the distance from the

central point as well as the proportion of seed production per inflorescence for different pollination treatments were analyzed by one-way ANOVA followed by Duncan's multiple tests at the 0.05 significance level wherever needed. All the analyses were performed with SPSS 13.0.

3. Results

3.1. Floral phenology and morphological parameters

Different sites had different effects on the phenology of *S. anglica*. Flowering took place from June until September in

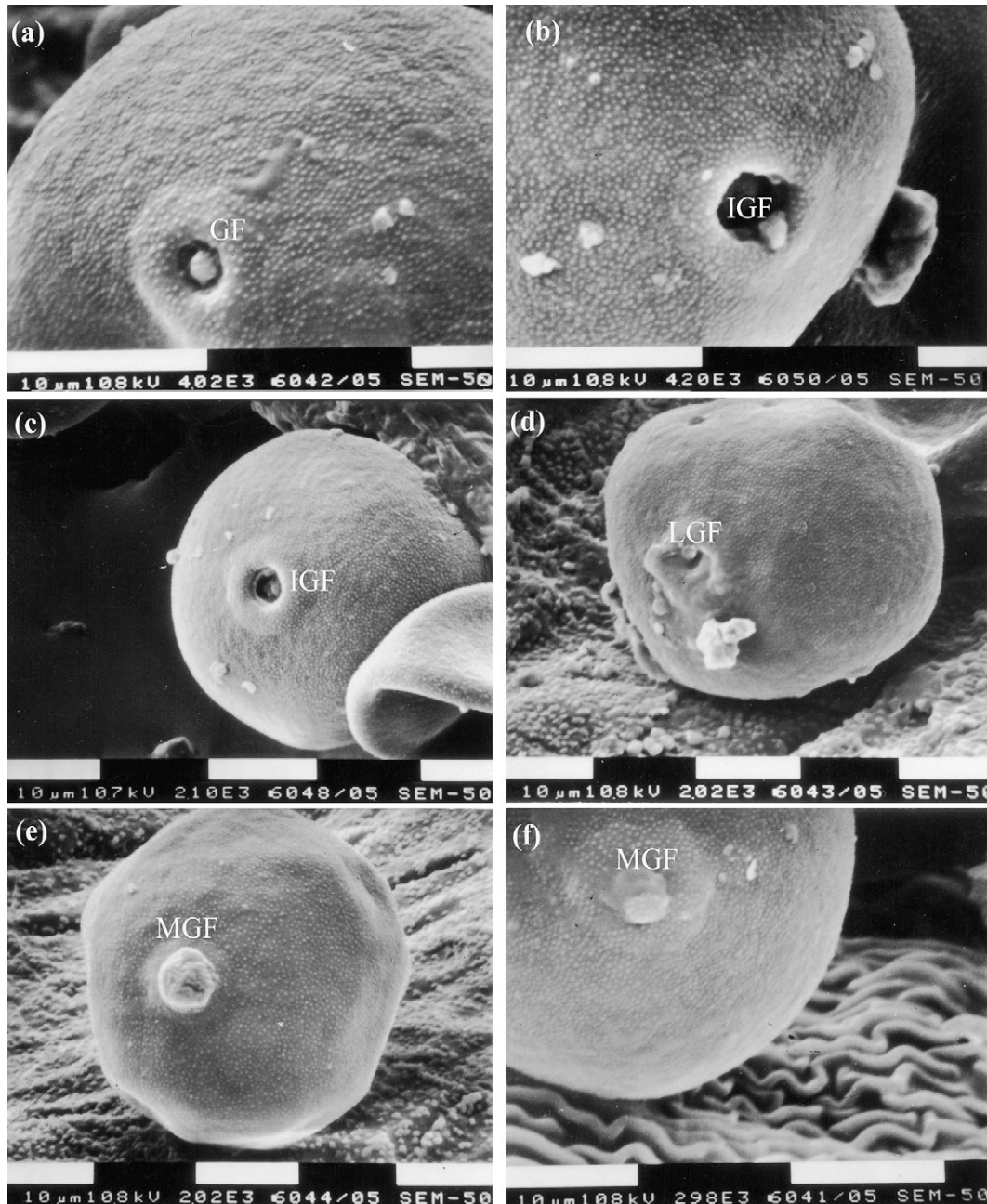


Fig. 1. The shape and irregularity of *S. anglica* pollen. (a) The regular pollen with a germination foramen (4200 \times); (b) and (c) the irregular pollen with irregular germination foramen, (b) 4200 \times and (c) 2100 \times ; (d) the irregular pollen with loss of germination foramen, (d) 2020 \times ; (e) and (f) the irregular pollen with the germination foramen and the pollen wall merged together, (e) 2020 \times and (f) 2980 \times . GF: germination foramen; LGF: loss of germination foramen; MGF: merged together the germination foramen and the pollen wall.

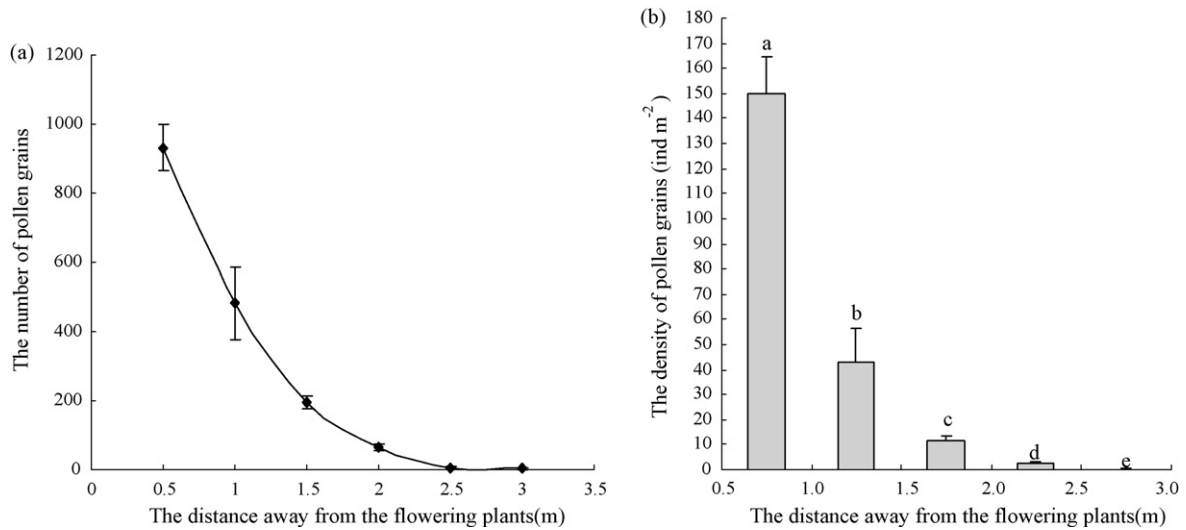


Fig. 2. Number and density of pollen grains delivery along the distance in the natural field. (a) The number and (b) the density. Mean \pm S.E., $n = 25$ (five patches by five directions). The wind speed during trials was $2.95 \pm 0.68 \text{ ms}^{-1}$. Different letters means significant difference ($p < 0.05$).

the field and from May to August in the greenhouse. Insignificant difference of floral parameters, such as the number of spikes per inflorescence and the number of spikelets per spike, occurred between two experimental sites (Table 1). There were three stamens and two pistils per flowerlet, which were fused for a short distance at the base. The stigmas were often large and of feather that emerged from the glumes. The inflorescence of *S. anglica* was a panicle consisting of 2.2 ± 0.5 or 2.2 ± 0.6 spikes ($n = 10$) which carried 9.8 ± 0.77 or 10.3 ± 0.93 sessile flattened spikelets arranged alternately in two rows, for the field and greenhouse, respectively (Table 1). The flowerlets usually consisted of one glume and one palea, with the length of the glume being 1.88 ± 0.06 cm in the field and 1.78 ± 0.06 cm in the greenhouse ($n = 10$).

The flower was protogynous. In both the field and the greenhouse, spikes of the inflorescence emerged from the sheath and the pistils of the flowers protruded, increasing from the sheath to 1.41 ± 0.31 cm or 1.19 ± 0.30 cm in length (Table 1; $n = 10$) and persisted as such for 4 days. The stamens

emerged on the fifth day. After the anthers appeared, they became completely free from the glumes within 1.5 h, after which the filaments continued to elongate. Their final length measured 1.42 ± 0.34 cm or 1.16 ± 0.27 cm ($n = 10$).

3.2. Pollen production, viability and irregular structure

The mean number of pollen grains produced per anther, P/O and pollen viability ratio per inflorescence in field and greenhouse were 771 ± 70 versus 509 ± 44 ; 2312 ± 209.8 versus 1528 ± 131.8 ; 0.282 ± 0.019 versus 0.208 ± 0.009 , respectively (Table 1). There was significant difference in the parameters among the different sites ($p < 0.05$) (Table 1).

The pollen shape was round for both the regular and irregular pollen (Fig. 1). The shape was spherical with a germination foramen (Fig. 1a). There were some irregular phenomena (Fig. 1). Some pollen had an irregular germination foramen (Fig. 1b and c); some completely lost germination foramen (Fig. 1d), and some lost viability due to a fusion of the

Table 2
Two-way ANOVA of the effect of time and pollination way on the pollen parameters in field experiment

Sources	Variables	d.f.	MS	F	p
Pollination way	The amount of pollen grain on stigma	1	1150.235	15.612	0.0003**
	The amount of germinated pollen on stigma	1	25.389	10.202	0.0026**
	The amount of abnormal pollen	1	10.225	9.676	0.0032**
	The percentage of abnormal pollen	1	0.024	0.673	0.4164
Time	The amount of pollen grain on stigma	5	1052.789	14.289	0.000**
	The amount of germinated pollen on stigma	5	95.462	38.359	0.000**
	The amount of abnormal pollen	5	37.791	35.759	0.000**
	The percentage of abnormal pollen	5	0.493	14.100	0.000**
Pollination way \times time	The amount of pollen grain on stigma	5	131.416	1.7837	0.036*
	The amount of germinated pollen on stigma	5	5.891	2.367	0.005**
	The amount of abnormal pollen	5	4.136	3.913	0.054
	The percentage of abnormal pollen	5	0.093	2.670	0.034*

* $p < 0.05$.

** $p < 0.05$.

germination foramen and the pollen wall (Fig. 1e and f). The percentage of irregular, non-viable pollen was $37.5 \pm 0.03\%$ and $33.1 \pm 6.9\%$ in field and greenhouse, respectively (Table 1).

3.3. Pollination distance and the effect of different manners of pollination

The amount of pollen decreased along the distance from the flowering plants, and no pollen was detected at the distance of 3.0 m (Fig. 2a). The pollen density was about 150 pollen grains/m² when collected at less than 1 m distance from the flowering plants, then significantly decreased to 42 pollen grains/m² in the range of 1.0–1.5 m distance (Fig. 2b).

Although the number of pollen tubes could not be precisely quantified, clear differences were observed in relation to the differing times and manners of pollination. The amount of pollen on the stigma, the amount of germinated pollen on the stigma and the amount of abnormal pollen were the factors most significantly affected by the time or manners of pollination. However, the cross-action weakened the independent effect on the amount of pollen grain on stigma, the amount of abnormal pollen and the percentage of abnormal pollen (Table 2). The amount of pollen grains on the stigma, the amount of germinated pollen grains and the amount of abnormal tubes increased significantly from 15 min to 75 min and dropped after that time in both self-pollination and cross-pollination (Fig. 3a–c). The highest amount of pollen grains on the stigma was 73 ± 6 and 34 ± 2 for cross-pollination and self-pollination, respectively, while the highest amount of germinated pollen grains and of abnormal tubes were 14 ± 1 versus 7 ± 1 , and 7 ± 1 versus 4 ± 1 , respectively. Unlike these

three parameters, the ratio of abnormal pollen tubes 30 min after pollination was high for both cross-pollination and self-pollination (Fig. 3d).

3.4. Differences in pollen-tube growth and the proportion of seed set

The pathway of pollen-tube growth to the embryo sac is shown in Figs. 4 and 5. During cross-pollination experiments, pollen landing on the stigma quickly hydrated and pollen tubes emerged from the pollen aperture adjacent to the stigma surface within 15 min (Fig. 4a). Pollen exhibiting self-pollination, however, germinated more slowly than did those exhibiting cross-pollination within the same time frame of 15 min (Figs. 4b and 5b). Pollen tubes passed through the intercellular spaces between papillae, and grew along the surface of the hollow transmitting tissues of the style. In the transmitting tissue, pollen tubes varied in width and in number of callose plugs (Fig. 4c). Abnormalities of pollen-tube growth such as mending tubes, forked and swollen tips, and curling were found at most collection times after the pollen germinated (Fig. 4d and e). Between one and two pollen tubes per flowerlet reached an embryo sac by cross-pollination either in the greenhouse or in the natural marsh (Fig. 4f). Hardly any pollen tubes grew to an embryo sac to complete self-pollination (Fig. 5f), but some pollen tubes were able to grow to a length of 75 min found self-pollination (Fig. 5e).

Different pollination treatments had significant effects on the proportion of seed production per inflorescence. The percentage of cross-pollinated seeds with $21.9 \pm 6.7\%$ was significantly higher than those of self-pollinated and naturally

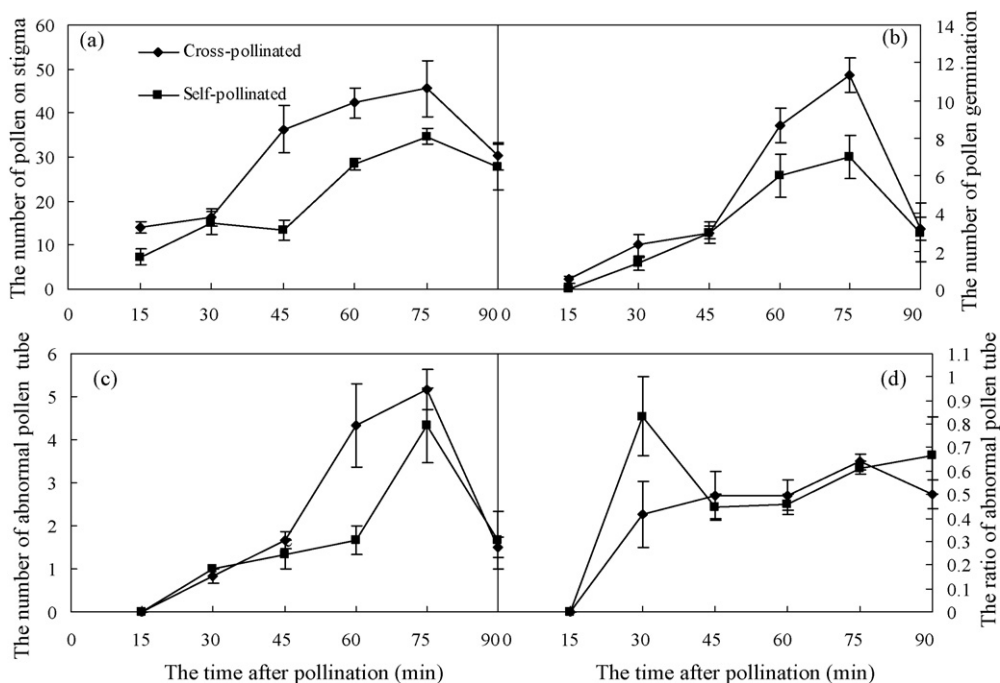


Fig. 3. Variation of pollen number and pollen tube at different time after cross-pollination or self-pollination. The amount of pollen grain (a), the amount of pollen germination (b), the amount of abnormal pollen tube (c) and the ratio of abnormal pollen tube (d) (mean \pm S.E.) ($n = 5$).

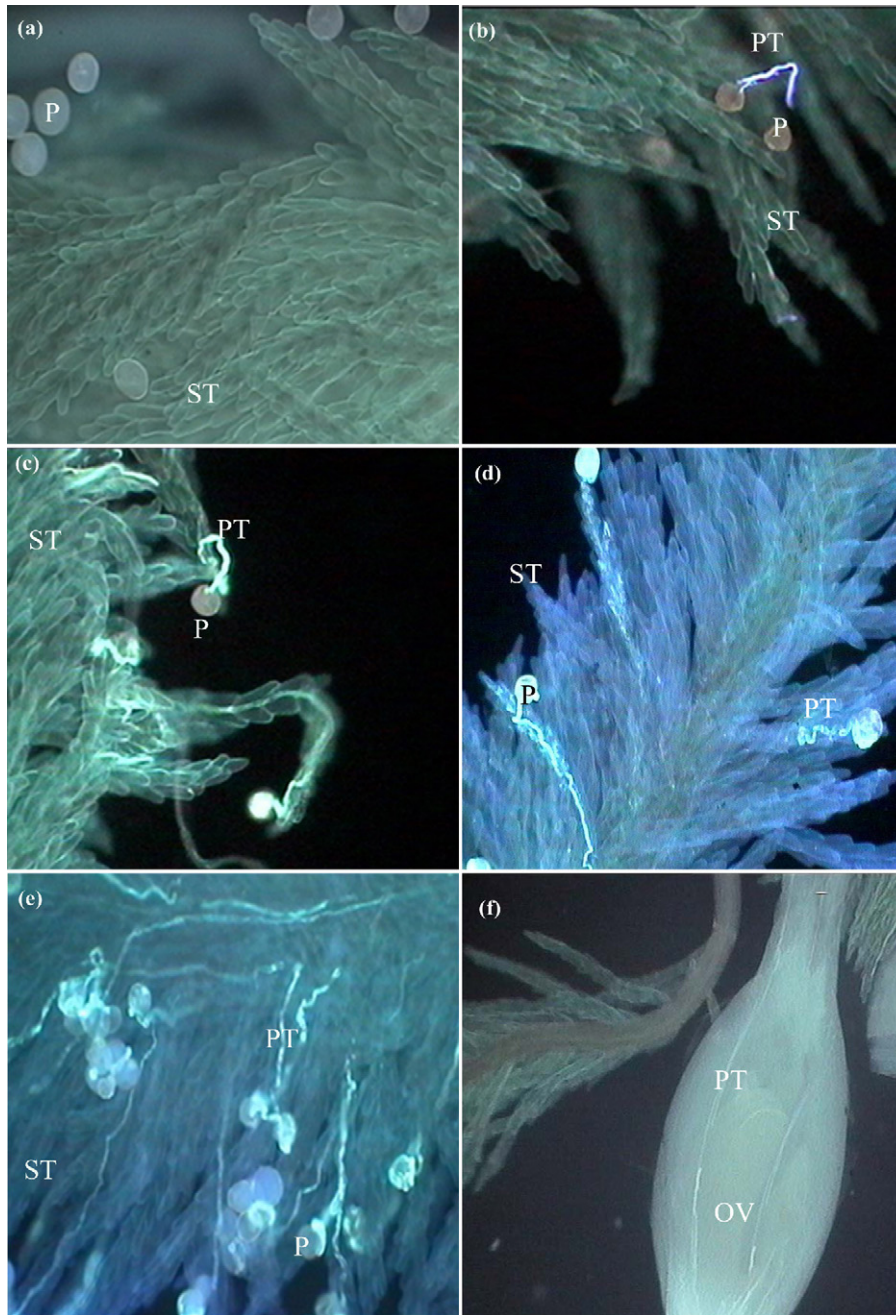


Fig. 4. Epi-fluorescence micrographs of pistils following cross-pollination in natural marsh. Panels (a)–(f) shows the pistil, respectively at 15 min, 30 min, 45 min, 60 min, 75 min and 90 min after pollination, there is one to two pollen tubes grown to the ovary. All pictures were multiplied by 10×10 times. Pistils were stained with decolourized aniline blue to localize callose. P: pollen; PT: pollen tubes; ST: stigma; OV: ovary.

pollinated seeds, but there was no distinct difference between the self-pollinated and natural-pollinated seeds (Fig. 6).

4. Discussion

4.1. Floral morphology

Dichogamy is assumed to be a common method involved in avoidance of self-fertilization [40], including protogynous and protandry. *S. anglica* was a hermaphroditic flower with some temporal separation of male and female functions (our data).

The flower was protogynous, and the stigma had appeared for 4 days before the anther's dehiscence. In fact, protogynous and protandry may serve to prolong pistil or pollen presentations, avoid pollen–stigma interference, and provide an optimal position for pollen dispatch and reception [41]. In *S. anglica*, however, the protogynous flowers still allow for very weak self-pollination (Figs. 5 and 6). Probably protogynous for *S. anglica* was one of factors that induced fewer pollen grains on the stigma. Different sites had no significant effect on the floral parameters (Table 1) and the transplant to the greenhouse did not help to restore the sexual reproduction ability of the species.

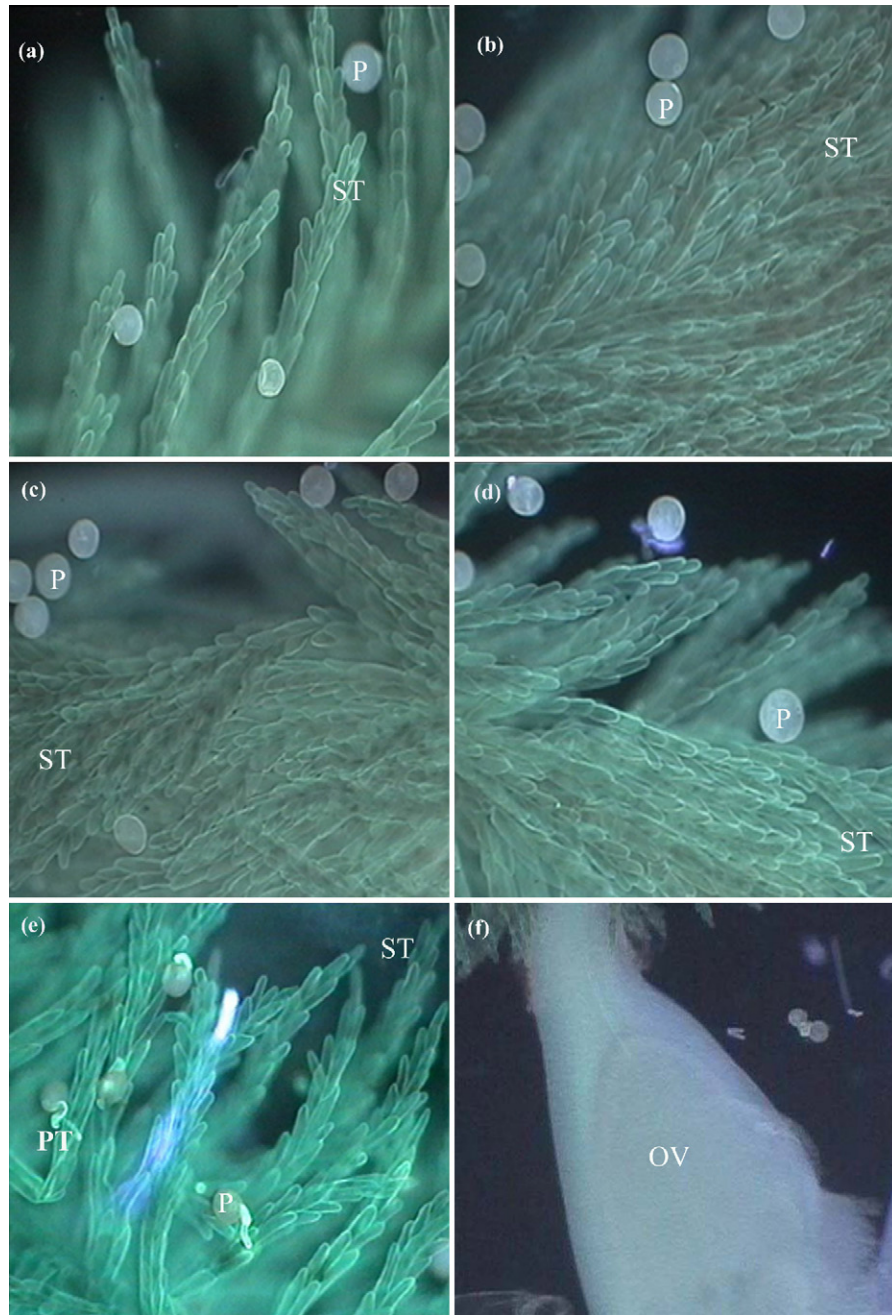


Fig. 5. Epi-fluorescence micrographs of pistils following self-pollination in natural marsh. Panels (a)–(f) shows the pistil, respectively at 15 min, 30 min, 45 min, 60 min, 75 min and 90 min after pollination, there is no pollen tube grown to the ovary. Pistils were stained with decolourized aniline blue to localize callose. P: pollen; PT: pollen tubes; ST: stigma; OV: ovary.

4.2. Pollen and pollination

Pollination is the primary step in seed formation. Previous studies have shown that pollination failure could occur at any step in the dispersal process and at several different levels [42]. Increased risk of pollination failure is associated with several problems, namely: pollen arriving either in prohibitively small quantities, too long after a certain period of time, or too mixed in composition, poor pollen quality [43], and a lack of pollinators [44]. Pollination failure also increased when the population was too sparse, too small in number, too

genetically uniform, too fragmented, or under rapid modification [42,45]. These criteria were especially debilitating for self-incompatibility plants [46]. Pollen shape of *S. anglica* is rounded and the size is adapted for easy liberation from the anthers by moderate wind [47]. P/O of *S. anglica* was the characteristic of wind pollination. However, the pollen could not travel more than 3 m on the Chinese coast (Fig. 2). Although *S. anglica* had a long anthesis period from June until September in the field patch, the number of flowering plants at the same time was very low. In natural patches, there was only one flowering plant per 114 m², and

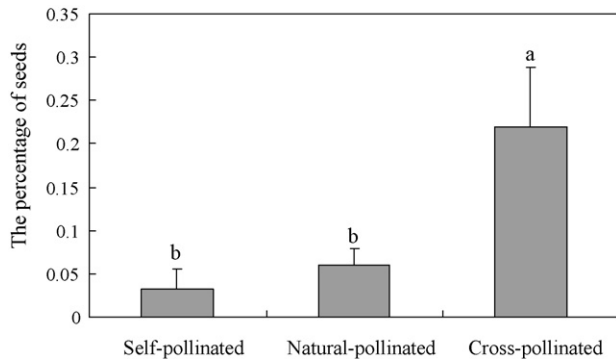


Fig. 6. The different seed production ratio per inflorescence among different pollination ways. Mean \pm S.E.; $n = 10$; different letters mean significant difference ($p < 0.05$).

we could confer that the stigma could barely receive cross pollens from farther distances.

Fates of flowers include flower abscission, seed abortion and seed production. This is determined by the pollen resource, the pollinated flower and available resources [48]. Many ecological and evolutionary hypotheses have been proposed to explain excessive flower production and the low ratios of fruit to flower, such as the pollen limitation hypothesis, the resource limitation hypothesis and the pollen donation hypothesis [49,50]. Seed set was pollen-limited or pollinator-limited when small amounts of pollen were deposited on stigmas [15,38]. Pollen limitation was probably caused by habitat fragmentation [51–54]. For *S. anglica*, the pollen on the stigma of manual cross-pollination equaled almost 72 pollen grains; however it was not more than this number during natural pollination. In addition, there was low pollen viability, since some irregular pollen and pollen tubes could not germinate or grow through to the ovules. It could be concluded that the low seed set of *S. anglica* was induced by pollen limitation and poor pollen quality.

For a good seed set, it is important to ensure that excessive pollen relative to the number of ovules is necessary to allow for pollen-tube competition [55]. There are many reports suggesting that pollen competition may be an important component of natural selection by the way of the process of gametophytic selection [19,56]. Seeds produced under intensive pollen-tube competition had a better germination ratio and better seedling growth than those produced with little or no pollen-tube competition [20]. In *S. anglica*, there was little pollen-tube competition because of insufficient pollination and thus the seed set was very low.

4.3. Pollen-tube growth and weak self-incompatibility

The different lengths of time at which pollen tubes grow through the style to the ovule may take result in the taking in of angiosperms. Some plants took 11–24 h for both self- and cross-pollen tubes to reach ovules [57]; some even took 2–6 days [21,58]. Ten or more days after pollination, both self- and cross-pollen tubes reached the lower end of the carpel [39]. In *S. anglica*, the pollen tube grew particularly fast. Almost 50% of pollen tubes in cross-pollinated flowers had grown to the base

of the style by 90 min. In self-pollinated plants, however, the pollen tube was hardly as successful.

Weak self-incompatibility may be one factor inducing the low seed set of *S. anglica*. Self-incompatibility could act in the stigma, style or ovary, or even during development of the embryo [59]. A number of the pollen tubes in self-pollinated flowers or afflicted with gametophytic self-incompatibility terminated in the style [58], but most were inhibited in the ovary [35]. A different mechanism could perhaps explain the phenomenon in cases of gametophytic self-incompatibility [60,61]. It was reported that *S. anglica* could be self- or cross-fertilized [34]. The fluorescent studies of pollen tubes in the pistil demonstrated that the stigma was in pollen recognition during both self- and cross-pollination. There was little to no space competition among pollen tubes. Although there were some pollen tubes exhibiting abnormality such as mending tubes, irregular tubes, forked and swollen tips and curling tubes, one or two pollen tubes still entered the micropyle for cross-pollination (Fig. 5f). Self-pollinated individuals could germinate on the stigma and slowly grow throughout the style. However, very few pollen tubes reached the micropyle. It is possible that *S. anglica* was weak self-incompatibility.

4.4. Environmental factors in relation to seed set

There were some unfavorable abiotic factors that may have affected seed set. Temperature was particularly important in determining the effectiveness of pollination, the stigma receptivity [47], the pollen germination and the pollen-tube growth. These factors in turn affect fertilization and fruit set. The optimum temperature for pollen germination and pollen-tube growth is about 25–30.7 °C [62,63]. Extreme temperatures could reduce pollination and pollen growth and induce seed abortion, thus eliminating or diminishing fruit set [62,64]. In Chinese *S. anglica*, different sites had a different influence upon the floral phenology, which is a phenomenon consistent with the previous research [65]. The unfavorable temperature was likely induced by the micro-climate variation, since the average temperature in the greenhouse was between 26.3 °C and 31.7 °C, and that of the natural field was 21.6–26.7 °C during the experimental period. The highest temperature during the June, July and August experimental period was 37.2 °C, 38.4 °C and 36.2 °C, respectively, and the average rainfall during that time was 464 mm, 598 mm and 180 mm, respectively in the natural field. The high temperature and humidity would make for less effective pollination or pollen germination, thus reducing seed set.

4.5. Seed abortion and dieback

There are some other reasons for low seed set beside those mentioned above. Inbreeding depression [27], competition for maternal resources [13] and the stigmatic embryos of earlier fertilization producing some chemicals [66] could also explain high rates of seed abortion. On Chinese *S. anglica*, high seed abortion in natural populations is mainly due to protogynous,

poor pollen quality, pollen limitation on the stigma, and irregularly shaped pollen or pollen tubes. In this experiment it is likely that unfavorable abiotic factors such as high temperatures and rain inhibited the pollination and pollen germination on the stigma. All these factors would induce the high seed abortion and low seed set ratio.

S. anglica's failure to reproduce sexually had a great effect on its natural population on the Chinese coast. Meanwhile, human disturbance led to the fragmentation of populations into small, isolated remnants, which also reduced genetic drift and resulted in low seed production [67,68]. After these events, population size, floral display size and distance between populations began to affect the seed set and gene flow [44] leaving the population with a severe lack of genetic diversity. Decreased genetic diversity coupled with the inhibition of gene flow between populations resulted in a very low amount of viable seed sets [69]. Thus, the Chinese population of *S. anglica* mainly relied on asexual reproduction for generations and the species was relatively genetically uniform. The ability to adapt to new conditions was also reduced, indicating that a low seed set ratio was one of the important reasons that induced the dieback of *S. anglica* in China.

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