Floral Structure and Pollination in Relation to Fruit Set in Cynanchum otophyllum Schneid

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Abstract—We studied the floral morphology and floral biology of Cynanchum otophyllum Schneid in experimental plots and field sites. Some observations were augmented by laboratory studies of floral traits, including scanning electron microscopy and light microscopy. The flower was characterized by a staminal corona. The pollinia were lodged in sacs on each side of the stigma and needed pollen vector for fruit production. C. otophyllum has characteristics similar to bee-pollinated plants. Pollinaria removal and pollinia insertion rates were low at 5.4% and 0.45%, respectively. The fruit set was only 2.2% in natural conditions and 0.45%, respectively. The fruit set was only 2.2% in natural conditions. The flowering span of C. otophyllum was about 3 months, and the functional longevity of individual flowers was 6-8 days. The extended period may be related to the relatively low levels of effective pollinator activity. The flowers were self-incompatible. Umbels displayed open flowers for 9-10 days, and there was a large overlap in flowering time within and among inflorescences in a single plant. Therefore, a high level of self-pollination is possible. From the significant increases in fruit set in cross-pollinated flowers (12.6%) compared with self-pollinated flowers (1.52%), the low fruit set in C. otophyllum could be partially explained by pollen limitation.

Keywords—Cynanchum otophyllum; floral biology; honeybees; pollination; fruit set

0 INTRODUCTION

The complex floral morphologies found in Asclepiadaceae have intrigued botanists for two centuries [1]. Most data for Asclepiadaceae have been principally accumulated from the observations of Asclepias in North America [2]. Thus, Ollerton and Liede [3] considered that overdependence on information about Asclepias could bias our conclusions about Asclepiadaceae as a whole.

Asclepiadaceae are characterized by low fruit set, typically averaging 1%-5% [2]. Cynanchum is a large genus of some 400 described species with a tropical and subtropical distribution in Africa, Madagascar, Australia, North and South America, and some parts of Asia [4]. Cynanchum is characterized by a staminal corona originating from a ring-shaped meristem [5] that can be extremely variable in shape and relationship to the gynostegium. Observations in Cynanchum are still scarce [3].

C. otophyllum is a medicinal plant endemic to China which produces bountiful flowers but not all of them mature into fruits [6]. This is the bottleneck restricting their cultivation. We studied the species with regard to flowering phenology, pollination system, and the relationship between floral structure and function of C. otophyllum in Yunnan, China. We addressed the following three points: (1) What kind of pollination system exists in C. otophyllum; (2) What floral characters of the flowers are correlated to pollination in C. otophyllum; and (3) What causes the formation of low fruit set in C. otophyllum.

1 MATERIALS AND METHODS

1.1 Study area

We established and monitored two experimental areas: A and B. Area A was located on a hill with yellow soil near the town of Longchang, Xianwei County, Yunnan Province (104°09' E, 26°11' N). The climate is subtropical with an annual average temperature of 14.1 °C. During the study period, rainfall was 45–65 mm, mean temperature was 21.6 °C (18.5–28.5 °C), and RH was 61.6% (59.8%–64.3%) at 0700 h. C. otophyllum plants grew in a dry area, covering about 3000 m² and usually thriving in patches. The surrounding landscape was a mixture of small woodlots and agricultural crops. There were about 500 plants in the whole area. Area B included plants grown from seeds collected in the area and transplanted to the Botany Experimental Field of Kunming University in early March 2006. A voucher specimen from the population of C. otophyllum studied was housed in Kunming University. Voucher specimens of insect species observed were also deposited in Kunming University.

1.2 Floral morphology and anatomy

We picked some mature flowers and immediately fixed them in FAA for morphological and anatomical examinations. Some flowers were sectioned in paraplast and stained with either hematoxyline or safranine and observed under an Olympus BX60 microscope. We also compared self- and cross-pollen tube growth in hand-pollinated flowers using fluorescence microscope (Martin 1959). These flowers were fixed 72 h after pollination in a solution of 3:1 ethanol: acetic acid for at least 24 h and sectioned. All microtome sections stained with decolorized aniline blue for 30 min. The stained pollen tubes fluoresced brightly when viewed with epifluorescence optics on an Olympus BX60 microscope. Critical point dried samples were examined through scanning electron spectroscopy (SEM).

1.3 Flowering phenology

We determined the flowering patterns from weekly counts of the number of umbels with open flowers in field
populations from July to October 2006 and 2007. In Areas A and B, 86 and 74 plants, respectively, were individually tagged with thin, red thread. Opening and wilting dates for each were recorded during the flowering season (2 July to 6 October 2006). In these two areas, the flowering dynamics of *C. otophyllum* population were recorded. More detailed observations were made in Area B from 23 inflorescences tagged while in bud, each part consisting of 2–5 flowers depending on inflorescence size. Every 3 h, we observed and recorded floral traits including the timing and duration of flowering.

1.4 Hand pollination

On 17–19 July, 23–25 August, and 4–6 September 2006, 3–5 flowers at a single node on each of the 60 plants were randomly assigned to the following treatment: (a) bagged without pollination; (b) emasculation, i.e., removal of all five pollinarium; (c) self-pollen, i.e., emasculation and insertion of pollinaria from another flower of the same plant; (d) cross-pollen, i.e., emasculation and insertion of pollinaria from another plant, and all the flowers were bagged before flowering and bagged again after treatment except the bagged without pollination; and (e) unmanipulated control. In the self- and cross-pollination treatments, the pollinarium was removed by inserting an entomological pin beneath the corpuscular. The removed pollinaria were then inserted in one of the five nectar-filled cavities lying directly beneath the corpuscular. All other flower buds were removed from the plants as they formed so that resources would be allocated to the experimental flowers. We recorded the state of follicle initiation and maturation every 3 days. All initiated follicles were characterized by visibly enlarged ovaries. Fully developed follicles were considered mature when no additional enlargement occurred during three consecutive weeks.

1.5 Pollinator and natural pollination efficiency

Pollinator observations were made for 5 days in Area A. Observations were made between 0800 and 0630 h. The behaviors of visitors were recorded, and some insects were collected for later identification or examination upon receipt of the pollen. A total of 400 flowers were checked for the number of removed pollinaria and inserted pollinia under a dissecting microscope.

1.6 Statistical analyses

Differences in means and standard deviations for the flowering span of individually marked flowers and umbels at different times and conditions were statistically tested with Student’s t-test [7].

2 RESULTS

2.1 Functional floral structure in relation to pollination

*C. otophyllum* (Fig. 1: A) is a perennial herbaceous vine that climbs to a height of 1–3 m (1.62 m on average, n=66) in the growing season. Inflorescences are umbel-like, producing 8–36 flowers (25 flowers on average, n=72). The flat, star-shaped flowers are small (up to 7 mm in diameter) with very pale green petals and a distinctive, tall white corona (Fig. 1: B). The flower consists of five parts (Fig. 1: B), and each part has a stigma and a pair of pollinia lodged in sacs on each side (Fig. C, E). The brown corpuscular of five pollinaria are easy to see against the yellow background of the central gyrostegium (Fig. 1: B). Nectar is often visible at the base of the petals. The flowers have a sweet, fruity odor.

The perianth consists of five sepals surrounding five petals (Fig. 1: B). The corona is inserted at the base of the gyrostegium (Fig. 1: B), which is membranous and deeply divided into five sections. The corona encircles the gyrostegium, the union of the stamens with the fleshy stigma head at the flower's center (Fig. 1: B). The gyrostegium consists of two pistils attached to the gyrostegium at the point of connection between the style and the stigma head (Fig. 1: C). The stigma head is probably stilar in origin because the true stigmatic surfaces are enclosed within the stigmatic chambers between adjacent anthers (Fig. 1: D).

The androecium consists of five stamens whose filaments are fused to form the column (Fig. 1: C). Each anther is bicarpellate (Fig. 1: E) and tipped with a white outgrowth of the connective that partially covers the stigma head. Each anther sac encloses a pollinium (Fig. 1: E), where approximately 170–190 pollen grains are held together by a waxy coating. The pollinia of each pair are connected by a thin band called translator arms to a brown, tubular structure called corpusculum on top of the stigmatic slit (Fig. 1: C, E). The entire pollination unit (i.e., two pollinia, two translator arms, and a corpusculum) has the general shape of a wishbone and is called a pollinarium (Fig. 1: C). The corneous margins of two adjacent anthers form the anther slit (guide rails), which provides the narrow entrance to the stigmatic chamber (Fig. 1: F). The corpusculum joining adjacent pollinia of different anthers is directly seated above the alar slit (Fig. 1: C, F).

Nectar serves as the pollinator attractant and is secreted by the basal glands at the base of the sepals (Fig. 1: J). The nectar accumulates in the opening of flower as well as in the stigmatic chambers where the nectar is removed by pollinators (Fig. 1: H). Often part of the tarsus and mouthparts of the pollinators, enters the opening between the corneous anther slit. Because the upper edges of the anther slit overlap the corpusculum (Fig. 1: C, F), with the upward pull of the insect, the tarsus or mouthparts become wedged in the groove of the corpusculum. When a pollinator then leaves, the pollinia are pulled from their sacs and the entire pollinarium is carried away (Fig. 1: I). When an insect carrying a pollinarium subsequently visits another flower, one of the pollinia may enter the stigmatic slit (Fig. 1: K). The bristles (Fig. 1: G) beneath the edges of the anther may prevent further removal of the pollinium in situ, so the inserted pollinium fills almost the upper portion of the stigmatic chamber (Fig. 1: K).

According to serial sections of hand-pollinated flowers, no differences were detected in the appearance of self- versus outcross-pollen tubes up to 72 hr after pollination. In both pollen types, abundant pollen tubes penetrate through the joined point (Fig. 1: L) of the two styles and then follow the direction of the transmitting tissue entering one ovary of the bicapellar pistil (Fig. 1: M) in most of the flowers, but some pollen tubes enter another ovary in some flowers. They grow
over the surface of the ovules and penetrate through the micropyles (Fig.1: N).

2.2 Flowering phenology and longevity of individual flowers

In Area A, *C. otophyllum* started to bloom in mid-July, and its flowering period continued until early October. Each plant produced hundreds of flowers over 10–12 weeks. The flowering span of *C. otophyllum* was about 90 days. The flowers began to bloom on 16 July, and the number of blooming flowers was high in the period of 12 August to 15 September. Afterwards, the number of blooming flowers gradually decreased until all flowers disappeared on 6 October.
The latest of the same plant began to flower on 30 September. In Area B, although the time of peaking and initial flowering was 1–2 weeks earlier than in Area A, the flowering phenology was closely congruent with that in Area A in the same year. The flowering phenology of *C. otophyllum* population in Areas A and B in 2006 is presented in Fig. 2.

Fig. 2 Blooming and wilting patterns of *C. otophyllum* in two populations in 2006

The duration of the flowers of an umbel remaining open and available to pollinators seasonally varied. Flowers remained open slightly longer in umbels produced earlier in the year than in late-flowering umbels in Area B. Each umbel displayed open flowers for approximately 10 days (Table 1). Flowers of the early and middle stages had 7–8 days’ duration, whereas the late ones had just 6–7 days. Nectar was produced in the first 4 days, but secretion on the surface of the gynostegium was scarcely seen.

### Table 1 Means and standard deviations for flowering span of individually marked flowers and umbels of *C. otophyllum* in experimental Area B, 2007

<table>
<thead>
<tr>
<th>Sampling date</th>
<th>7/22</th>
<th>8/15</th>
<th>9/20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days open per flower</td>
<td>8.08±0.14</td>
<td>7.82±0.42</td>
<td>7.53±0.71</td>
</tr>
<tr>
<td>Days open per umbel</td>
<td>10.26±0.21</td>
<td>9.89±0.65</td>
<td>9.65±0.63</td>
</tr>
<tr>
<td>Days between opening of first and last flower</td>
<td>2.25±1.09</td>
<td>2.06±0.82</td>
<td>1.87±0.56</td>
</tr>
<tr>
<td>n</td>
<td>35</td>
<td>35</td>
<td>32</td>
</tr>
</tbody>
</table>

2.3 Insect visitors and their behavior

Over 12 species of anthophilous insects belonging to Hymenoptera, Diptera, Coleoptera, Hemiptera, and Lepidoptera were recorded as flower visitors in Area A, but many of them only rested on the flower or gathered nectar. The flowers were regularly visited by a number of small bees.

The only insects carrying pollinaria of *C. otophyllum* were *Apis cerana*, which purposefully foraged between flowers on the same plant and then flew to another plant. As the bees landed on each flower, they grasped the cone-shaped corona with their legs and inserted their tongues into the opening on top of the corona (Fig. 1: G). The honeybees doing so would pick up and/or deposit pollinia using their mouth and legs. On 25 July, 50 honeybees were collected while visiting the flowers of *C. otophyllum*. We observed honeybees with pollinaria attached to their mouth and legs with up to three corpuscular in the case of one individual (Fig. 1: H). Wasps were occasionally observed to visit the flowers of *C. otophyllum* but were never observed to carry pollinaria. Visitation of beetle, ants, tumblebugs, and flies, although not pollinia transfer, were recorded as nectar gatherer.

2.4 Pollinaria removal and pollinia insertion

Natural pollination efficiency was determined by counting the number of pollinaria removed and pollinia inserted for 400 flowers. Only 108 of the 2000 pollinaria (400 flowers×5 pollinaria/flower) were removed (rate of pollinaria removal was 0.054±0.44), and only 18 of the 4000 available pollinia were inserted in the interstaminal slit (rate of pollinia insertion was 0.0045±0.23).

In the flowers whose pollinaria were removed, 23.9% had one pollinaria removed, 2.6% had two pollinaria removed, and 0.5% had three pollinaria removed. In the pollinia inserted flowers, there were at most two pollinia deposited in the interstaminal slit (Fig. 3).
2.5 Breeding system and fruit set

Based on the observations on the treatment experiment, no follicle formed in the flowers without artificial pollination (n=256), indicating the impossibility of automatic selfing. The absence of a pollen vector and no follicle formed in the flowers whose pollinaria were removed (n=186) indicated no agamogenesis occurred in *C. otophyllum*. Out of the 263 self-pollinated flowers, 14 visibly enlarged ovaries were initiated, but only 4 (1.52%) bore green follicles. Out of the 214 cross-pollinated flowers, 29 visibly enlarged ovaries were initiated and 27 (12.6%) bore green follicles. The controlled flowers (n=272) initiated 22 follicles but formed only 6 (2.2%) mature fruits (Table 2). Two of these fruits had two follicles, whereas the others only had one.

Table 2 Fruit formation number of *C. otophyllum* flowers

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of initiated follicles</th>
<th>Number of mature fruit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bagged without pollination</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pollinaria removed</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pollinaria removed, self-pollen added</td>
<td>14</td>
<td>4</td>
</tr>
<tr>
<td>Pollinaria removed, cross-pollen added</td>
<td>29</td>
<td>27</td>
</tr>
<tr>
<td>Unmanipulated control</td>
<td>22</td>
<td>6</td>
</tr>
</tbody>
</table>

3 DISCUSSION

3.1 Pollination system of *C. otophyllum*

In Asclepiadaceae, at least 8 pollination systems can be identified [3]. The results of the breeding and insect-exclusion experiments indicate that flowers of *C. otophyllum* are self-
incompatible and need pollen vector for fruit production. The overall fruit set from cross-pollinations was 12.6% versus 1.52% from self-pollinations. The finding of pollen-bearing honeybees (*Apis cerana*) strongly suggests that *C. otophyllum* has honeybee-pollinated flowers. Clearly, this species produces nectar in sufficient quantities to hold the attention of bees. In contrast, the most studied genus, *Asclepias*, is usually visited by a wide variety of insects [8]. The present results support that in *Cynanchum viminale*, a wide range of flower visitors is observed, but only very few carry pollinaria [9]. Only Ollerton and Liede [1] published data in *C. adalinae* subsp. *adalinae* on what appears to be specialized bee pollination in the genus. In this study, pollinaria were attached to both legs and mouth of insects. A similar phenomenon appeared in *C. caudatum* and *C. wifordii* [10]. Although wasps, beetle, ants, tumblebugs, and flies visited the flowers of *C. otophyllum*, the flowers of *C. otophyllum* are not morphologically adapted to them. The little variation in flower size is not suitable for the wasps’ legs or mouths to catch the corpusculum, which is inconsistent with reports in other milkweeds [11]. In Asclepiadaceae, the corona dose not only serves as an optical attractant for pollinators, but also as pollinator exclusion and manipulation of pollinator behaviour and positioning [11,12]. The beetle, ants, tumblebugs, and flies were not strong enough to remove the corpusculum, and thus some flies and ants were trapped in the corpusculum and died in the flowers. Liede [13] stated that guide rails play a crucial role in trapping the legs or proboscis of insects. If an insect is too small to remove the pollinaria, it becomes stuck in the guide rails and dies. Therefore, the guide rail also plays an important role in excluding unimportant pollinators [10]. Johnson and Steiner [14, 15] hypothesized a dominant view that pollination systems tend toward specialization. Our observations indicate that the flowers of *C. otophyllum* are functionally specialized.

3.2 Some phenomenon in cross-pollinated flowers

Although the fruit set is higher from hand cross-pollination (12.6%), it never approaches 100%. An unusual feature of milkweed reproduction is that germination of the pollen is in a nectar solution secreted within the stigmatic chamber [2]. In *Asclepias syriaca*, germination is inhibited by sucrose concentrations above 30% [16]. In *C. otophyllum*, we observed that the bagged flowers were full of nectar. The concentrated nectar within the stigmatic chamber may be the factor that inhibits pollen germination, resulting in the low fruit set in hand-pollinations.

Pollens from a single pollinium enter two ovaries in some flowers and form twin follicles in cross-pollinated flowers in *C. otophyllum*. Sage et al. [17] stated that three adjacent stigmatic chambers transmit pollen tubes to one of the two separate ovaries, and the remaining two chambers transmit pollen tubes to the second ovary in *Asclepias amplexicaulis*. Morse [18], however, observed the phenomenon that nearly 2% of all successful hand pollinations with a single pollinium produced twin follicles, indicating fertilization of both ovaries by pollen tubes from a single stigmatic chamber appeared in some flowers of *C. otophyllum*.

3.3 Flowering phenology and longevity of a single flower

Although the functional longevity of individual flowers was variable in different stages of flowering, its maximum value (8 days) can be regarded as physiological longevity of
the flower in *C. otophyllum*. This is an exceptionally long reproductive span compared with most temperate or tropical species studied. Janzen [19] showed, for example, that flowers of many tropical species pollinated by trap-lining insects or hummingbirds last only a day. Wyatt [20] speculated that the relative longevity of milkweed flowers might be related to the low efficiency of the pollinator. In *C. otophyllum*, the rate of pollinaria removal was 0.054±0.44 and the rate of pollinia insertion was 0.0045±0.23. The extended period of time that *C. otophyllum* flowers remain attractive may be related to the relatively low pollination efficiency observed in natural populations.

Long-lived umbels were associated with early flowering and, quite logically, greater floral longevity and reduced synchrony in anthesis. Differences were evident for early-versus late-season umbels in *C. otophyllum*. These observations are in agreement with those on *Platanthera bifolia* [21]. As suggested, floral senescence was probably connected with the reallocation of resources to developing seeds.

### 3.4 Causes of low fruit set

In many species of higher plants, very few fruits mature relative to the number of fertile flowers [22]. The fruit set of *C. otophyllum* is very low both in natural populations (2.2%) and in artificial pollination (12.6%). In an attempt to explain the phenomenon in natural populations in milkweeds, two hypotheses were developed [2]: (i) resources to mature fruits are limited, and (ii) an insufficient number of compatible pollinia reach the stigmatic chambers. In the experiment, we observed many of the fruits form only one follicle, with one carpel aborted. Even abortion of the apparently fertilized fruits is common. Queller [23], Willson and Price [24] reported the same phenomenon in some *Asclepias* species. The low ratio of mature fruits to flowers in *Asclepias* has most frequently been explained by postulating some form of competitive interaction among fertilized ovaries [25, 26, 27]. Wyatt [28] demonstrated that early competition among ovaries within umbels is a major factor resulting in reduced levels of fruit sets.

Pollinaria removal rate allows for an estimation of insect activity in Asclepiadoideae [25]. In *C. otophyllum*, the pollinaria removal rate was low with 5.4% and still lower to that showed by Wolff et al. [29] with flowers of *C. harlingii*. The remarkably complex and small-sized flowers of *C. otophyllum* drastically affect the pollinators’ effectiveness in removing the pollinia from a flower and inserting it into another flower. Although bees could catch up to three flower clusters (Fig. 1: I), the number of pollinia lodged in stigmatic chambers may not be sufficient. Another consequence of long reproductive spans for individual flowers is a large overlap in flowering times of different umbels in a single inflorescence (Table 1). This overlap in flowering times and the consequent increased level of self-pollination were further augmented by the synchronous opening of flowers in different stems of the same plant [21]. Meve et al. [30] demonstrated for *stapelia* that most of the (artificially crossed) species are self-sterile. In the self-pollinated and the unmanipulated control experiments for *C. otophyllum*, the number of initiated follicles was relatively high at 14 and 22, but the number of mature fruit decreased to 4 and 6 (Table 2). Insertion of pollinia from the same plant probably influenced the initiated follicles to form mature fruit. Considering the significant increases in fruit set in cross-pollinated flowers compared with self-pollinated flowers (Table 2), the low fruit set in *C. otophyllum* can be partially explained by pollen limitation. Evidence for simultaneous limitation by both pollen and resources in *C. otophyllum* will require additional exploration.

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