

# Effect of Resource Reallocation on Pollen Limitation and Reproductive Assurance of *Apocynum venetum* L. in an Arid Region

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## Abstract

Pollen limitation is considered to be a strong force driving the evolution of reproductive strategies in flowering plants. However, resource reallocation may occur among seeds because of variation in the quantity or quality of pollen received. We performed single-flower and whole-plant pollen supplementation and emasculation of flowers of *Apocynum venetum* L. to estimate pollen limitation and reproductive assurance. In addition, we calculated the visiting patterns of the pollinators, frequency of pollinators to estimate the effect of pollinator activity and further on the reproductive success of *A. venetum*. Our results indicated estimates from single-flower manipulations were not biased, closely resembling those from whole-plant manipulations. We found *Apis mellifera* was the dominant pollinator during the pollination process. In addition, insect pollination played a more important role in the outcrossing system, which could explain the differences in reproductive success. Our results also showed that autonomous self-pollination eliminates pollen limitation and provides reproductive assurance.

**Keywords:** resource reallocation, pollen limitation, reproductive assurance, pollinator, seed set

## 1. Introduction

In the past three decades, a handful studies have found a large proportion of flowers within a plant don't develop into fruits and seeds (Lloyd, 1980; Sutherland & Delph, 1984). Recent papers have indicated that angiosperms commonly mature fewer seeds than the number of flowers they produce, and the seed set is highly relevant to a plant's reproductive success (Burd, 1994; Guitian, 1993). Several hypotheses have been presented to explain this common phenomenon, especially pollen limitation. In flowering plants, pollen limitation is considered to be the dominate force driving the evolution of pollination strategies and reproductive success (Lloyd, 1992; Maurice & Fleming, 1995; Ashman et al., 2004). Pollen limitation, a decrease in potential plant reproduction due to inadequate pollen receipt, is ubiquitous across Angiosperms (Ashman et al., 2004; Knight et al., 2005). Pollen quantity limitation occurs when pollinators are scarce or ineffective (Gómez et al., 2010). Many empirical studies indicate the evolutionary outcome of pollen limitation is the evolution of self-fertilization because selfing elevates seed production and therefore provides reproductive assurance in poor pollination environments (Baker, 1955; Jain, 1976; Morgan & Wilson, 2005).

Many recent studies show that estimates of pollen limitation are often biased because plants may be able to reallocate resources among flowers or across years (Stephenson, 1981; Zimmerman & Pyke, 1988). Recent reviews indicate that the effect of reallocate resources on single-flower and whole-plant manipulations is different (Knight et al., 2006). In some studies, many flowers are implemented pollen for the entire lifetime of the plant, whereas in others, only a portion of the flowers during a plant's lifetime are supplemented (Knight et

al., 2006; Ryan & David, 2013). Whole-plant manipulations are often viewed as more accurate because resources cannot be differentially reallocated among fruits according to pollen quantity or quality (Wesselingh, 2007; Ryan & David, 2013).

*A. venetum* plays an important role in the maintenance of desert ecosystem stability because its root system is efficient in absorbing water, making it resist drought (Jiang & Li, 1997). In addition, *A. venetum* is of high economic value, and its flowers and roots contain cardiac glycosides which have been reported to help stable blood pressure (Ren et al., 2008). Therefore, understanding the mechanisms of flower and seed production is particularly important for increasing economic potential of *A. venetum* (Chen, 2015).

The general goal of this study is to describe effect of resource reallocation on pollen limitation and reproductive assurance in this ecologically important species. Our specific objectives are: (1) to determine the effect of pollen limitation on reproductive success throughout the seed set; (2) to examine pollinator visitation how affect the pollen limitation; and (3) to explore the effect of resource reallocation on pollen limitation and reproductive assurance when manipulations involve single flowers per plant and many flowers per plant. In addition, we examined the influence of self-pollination during the flowering process, explaining the important reproductive strategy.

## 2. Materials and Methods

### 2.1 Species

*A. venetum* is widely distributed in Northwest Gansu, East Qinghai and North Xinjiang. It is usually 1.5-3 m in height. For this species, the leaves are long oval and 1-5 cm long. *A. venetum* has cone cymes, bisexual flowers in purple or light red, five petals and five stamens (Lu et al., 2007). We observed 100 flowering individuals and the length of the corolla, stamens and pistil in mm was (Mean  $\pm$  SD):  $7.39 \pm 0.39$ ,  $2.43 \pm 0.20$  and  $2.16 \pm 0.18$ , respectively.

### 2.2 Study Area

The investigation was carried out in Linze Inland River Basin Comprehensive Research Station in Gansu province of China (between  $37^{\circ}50'-42^{\circ}40'N$  and  $100^{\circ}02'-100^{\circ}21'E$ ). Annual rainfall is about 117 mm, and the mean annual pan evaporation is 2390 mm. The six patches were 200-300 m away from each other and the average density of *A. venetum* in these patches is 20 individuals per 100 m<sup>2</sup>. In each patch, the flowering time of *A. venetum* and other species of plants do not overlap.

### 2.3 Pollen Limitation and Reproductive Assurance

To test whether the estimates of pollen limitation and reproductive assurance were dependent on the number of flowers manipulated per plant. The pollen-supplementation experiment was conducted in six studied patches from 2013 to 2015. In each patch, we marked 300 flowers at the closed bud stage and designated them into six treatments: (1) single flower un-manipulated (C treatment), flowers were marked in the same manner; (2) single flower emasculated (E treatment), the stamens of the flowers were removed before pollen liberation; (3) single flower supplemented (PA treatment), we hand-pollinated flowers by administering pollen which was collected from the newly opened flowers of donor individuals located at least 10 m distant from the receiving flowers; (4) all flowers un-manipulated (C treatment); (5) all flowers emasculated (E treatment); and (6) all flowers supplemented (PA treatment). In addition, these flowers were collected and the seeds were counted in the laboratory.

In this study, we used the seeds set to determine the magnitude of pollen limitation. The PL indices for the C treatment were calculated according to the following equation (Larson & Barrett, 2000):

$$PL_C = 1 - (RS_C/RS_{PA}) \quad (1)$$

Where  $RS_C$  is the seed set under the C treatment and  $RS_{PA}$  is the seed set under PA treatment. Positive value means result from higher reproductive success in PA than C indicates pollen limitation, while zero or negative values indicate no pollen limitation (Fernández et al., 2012).

### 2.4 Observations of the Pollinators

To determine the relationship between the pollinator visitation frequency and  $PL_C$  index, we conducted surveys of pollinators in six studied patches from 2013 to 2015. The quantity and identity of pollinators were recorded from May to July. In each patch, 100 flowers were tagged and repeatedly observed between 07:00 and 19:00. The pollinators collecting pollen and nectar were noted, and the duration of the pollinator visit was assessed using a DAT-recorder. The pollinators were captured using insect nets, and species of pollinators were identified by specialists. The presence of pollen grains adhering to their bodies was determined in the laboratory with a

stereomicroscope. The percent of inflorescences visited and the visitation frequency to flowers were recorded and calculated according to the following equations (Goverde et al., 2002):

$$\text{Percentage of inflorescences visited} = \frac{\text{Mean of inflorescences visited per dominant pollinator}}{\text{Total number of inflorescences in the patch}} \quad (2)$$

$$\text{Visitation frequency} = \frac{\text{Total number of visits to flowers in the patch per dominant pollinator}}{\text{Total number of flowers} \times \text{Observation time in the patch}} \quad (3)$$

### 2.5 Breeding System

To describe the breeding system of *A. venetum*, we randomly marked 300 flowers and designated them into five treatments between single-flower and whole-plant manipulations: (1) control (natural pollination); (2) manual self-pollination, flowers hand-pollinated with self-pollen and then bagged to prevent insect and wind pollination; (3) non-manipulated cross-pollination, where flowers were emasculated without a bag; (4) manual cross-pollination, pollen from different individuals was transferred to the stigma of emasculated flowers, and the inflorescences were bagged; and (5) emasculation and netting, the stamens of the flowers were removed before pollen liberation, flowers bagged with a fine 1 mm<sup>2</sup> mesh to prevent insect visitation. In addition, we had previously removed other blooming flowers to avoid a flower receiving its own pollen from another flower on the same plant. In October, we counted the number of seeds per fruit for each treatment in the laboratory.

### 2.6 Statistical Analysis

We used a type III model for analyses of variance (ANOVA, type III model), with size and treatment as the fixed factors to determine the effects on the percentage of visited inflorescences and the visitation frequency to flowers.

To compare the seed set between the pollen added and control treatments, we performed repeated measures analysis, using treatment as the within subject factor.

The Chi-square test was used to compare the proportion of flowers and seeds set among these treatments. All analyses were performed using the statistical software package SPSS 19.0 for Windows (SPSS Inc. Chicago, IL, USA).

## 3. Results

### 3.1 Pollen Limitation

In the single-flower manipulations, the seed set did differ significantly between the C and PA flowers, with 22.7 ± 3.2% in the C treatment, and 31.6 ± 3.8% in the PA treatment ( $P < 0.05$ ; Figure 1). In the whole-plant manipulations, a similar seed set by the flowers was also observed: 24.7 ± 2.9% in the C treatment, and 35.9 ± 4.3% in the PA treatment.

Pollen supplementation did significantly increase the seed set according to the comparison of flowers between the PA and C treatments ( $P < 0.05$ ; Table 1). The results of the PL indices indicated that pollen limitation was more severe in the whole-plant manipulations (PLc = 0.516 ± 0.031) than in the single-flower manipulations (PLc = 0.476 ± 0.026), but there was no significant difference between manipulations ( $P > 0.05$ ).

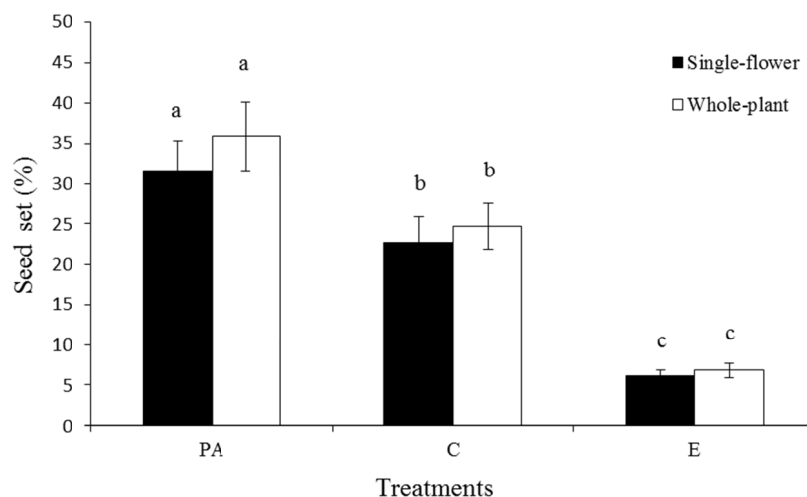


Figure 1. The mean seed set under different pollination treatments

Note. Black bars indicate seed set in the single-flower manipulations and white bars was seed set in whole-plant manipulations. Vertical bars denote standard errors. Pollen-supplementation treatments: A, pollen added; C, control; and E, emasculated.

Table 1. Effect of pollen supplementation treatments on *A. venetum* reproductive output

			Seed set			
			Single-flower		Whole-plant	
		df	F	P	F	P
PA vs. C	Treatment (T)	1	671.738	P < 0.01	1216.75	P < 0.01
	Patch (P)	5	2.157	0.29	1.37	0.321
	T × P	5	6.325	0.010	0.57	0.712
	PLc index		0.476 ± 0.026		0.516 ± 0.031	

Note. PA, pollen added treatment; and C, control treatment.

### 3.2 Reproductive Assurance

For single-flower manipulations, the seed set of C treatment, E treatment, and PA treatment was (Mean ± SD): 22.7 ± 3.2%, 6.1 ± 0.8%, and 31.6 ± 3.8% respectively. We found the significant effect on seeds set among the three treatments (P < 0.05; Figure 1).

For whole-plant manipulations, C treatment produced only 24.7 ± 2.9%, and the seed set was 6.8 ± 0.9% in the E treatment and 35.9 ± 4.3% in PA treatment, respectively. Our outcomes indicated the seed set of C and PA treatment was significantly higher than E treatment in both manipulations (P < 0.05). In *A. venetum*, we found self-pollination can serve as a mechanism to ensure reproduction regardless of the single-flower or whole-plant manipulation. In addition, the seed set of E treatment in the single-flower manipulations was no significant lower than that of the whole-plant manipulations (P > 0.05). There were no other significant differences among treatments or between manipulations.

### 3.3 Type and Visitation Frequency of Pollinators

In the studied patches, five species of pollinator were registered. *Apis mellifera* was the dominant pollinator because the bees visited the flowers from the time they opened until they closed, and visited more flowers per minute than other pollinators. In addition, *Ophion luteus*, *Anthidium septemspinosum*, *Episyrphus balteatus*, and *Pieris rapae* were also recorded and considered occasional pollinators. These pollinators collected pollen and nectar and transported the pollen from one flower to another, with activity peaking from 10:00 to 14:00 h. In total, the visiting patterns of 238 pollinators were recorded, and 137 pollinators belonged to *A. mellifera*. The remaining pollinators were other Hymenoptera (56), Lepidoptera (27) and Diptera (18). For *A. mellifera*, the percentage of visited inflorescences and the visitation frequency was significantly higher than that of other pollinators (P < 0.05; Figures 2 and 3).

These results showed the lowest and highest  $V_f$  of *A. mellifera* was  $3.57 \pm 0.4$  and  $5.43 \pm 0.6$ , respectively, and the  $PL_C$  index was significant difference interaction of the six patches ( $P < 0.05$ ; Figure 4). Our results suggested that the increase of visitation of pollinators could reduce the  $PL_C$  index.

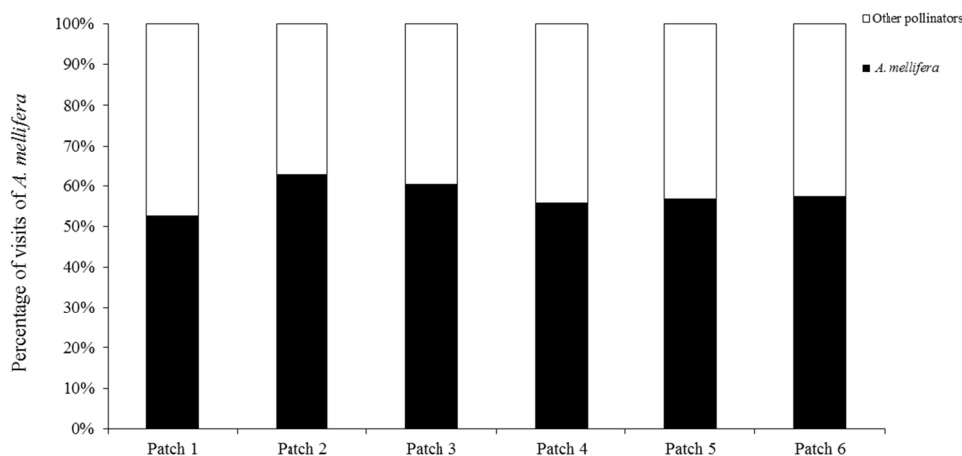


Figure 2. Percentage of visits of *A. mellifera* and other pollinators in six patched habitats

Note. Black bars indicate *A. mellifera* and white bars was other pollinators. The difference was significant between the *A. mellifera* and other pollinators ( $P < 0.05$ ).

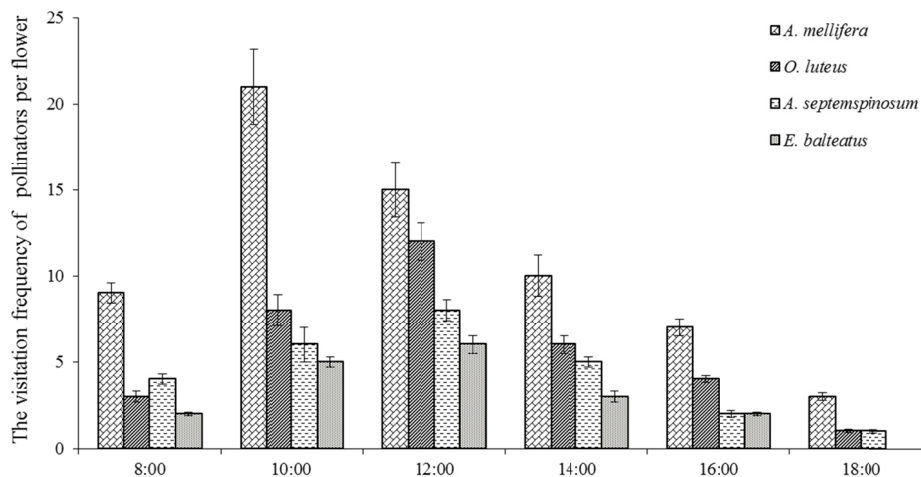


Figure 3. Frequency of pollinator visits over time to flower of *A. venetum*

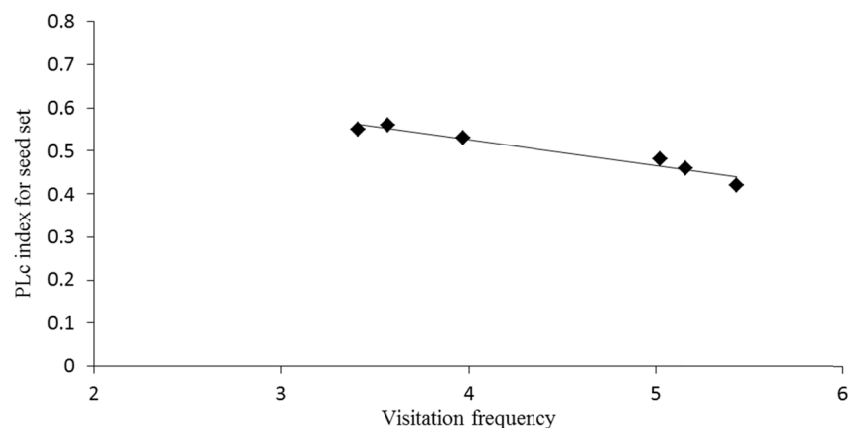


Figure 4. Relationships PL<sub>C</sub> index and visitation frequency of *A. venetum*

### 3.4 Breeding System

The seed set obtained in each pollination treatment is shown in Table 2. The seed sets of manual self-pollination treatment ( $32.8 \pm 3.5\%$  and  $7.6 \pm 1.2\%$ ) were clearly reduced in comparison to the seed sets of control treatment ( $23.5 \pm 2.1\%$  and  $37.2 \pm 3.8\%$ ), which resulted in significant differences in seed set between single-flower and whole-plant manipulations (Chi-square test,  $P < 0.05$ ). In both manipulations, the seed set of the manual cross-pollination treatment was  $6.9 \pm 0.8\%$  and  $7.6 \pm 1.2\%$ , respectively. These outcomes suggested that self-pollination successfully promoted the pollination efficiency, outcrossing played a complementary role in the breeding system.

Under natural conditions but in the absence of biotic pollinators, the seed set of emasculating and netting treatment was only  $3.6 \pm 0.4\%$  in the single-flower manipulations and  $4.7 \pm 0.5\%$  in the whole-plant manipulations. However, the flowers were able to produce more seeds when emasculated under natural conditions, and the seed set of the non-manipulated cross-pollination treatment was significantly higher than that of the emasculating treatments (Chi-square test,  $P < 0.05$ ). These outcomes indicated insect pollination played a more important role in the outcrossing system.

Table 2. Seed set of *A. venetum* under different treatments

Treatments	Seed set (%)	
	Single-flower	Whole-plant
Control	$23.5 \pm 2.1$	$26.3 \pm 2.8$
Manual cross-pollination	$6.9 \pm 0.8$	$7.6 \pm 1.2$
Non-manipulated cross-pollination	$4.3 \pm 0.5$	$5.2 \pm 0.6$
Manual self-pollination	$32.8 \pm 3.5$	$37.2 \pm 3.8$
Emasculated and netting	$3.6 \pm 0.4$	$4.7 \pm 0.5$

## 4. Discussion

### 4.1 Pollen Limitation and Pollinators

Pollen limitation may be caused by a decrease in pollen quantity or quality (Harder & Aizen, 2010). Pollen quantity may be reduced as a result of fewer pollinator visits or less pollen delivered per visit, and pollen quality can be reduced if self or otherwise incompatible pollen is delivered (Ashman et al., 2004). According to recent reviews, most pollen limitation occurs when pollinators are scarce or ineffective or pollinators when deposit incompatible pollen (Johnston, 1991; Gómez et al., 2010). In animal-pollinated plants, insufficient pollen deposition or deposition of low-quality pollen is mostly caused by pollinator assemblage characteristics, such as pollinator identity and behavior. Results from this study showed the bees was the most frequent pollinator, they landed on the stamens with their heads and extracted the nectar with their proboscis. In addition, the bees collected pollen from the anthers and stigma. The bees landed on the flowers to extract nectar their abdomens

were necessarily over the stigmas and pollinating the flowers because of their larger body size. More specifically, the bees visited flowers with a higher frequency as compared with the other pollinators because they were the most effective pollinators.

Many studies found pollen limitation to be associated with decreased visitation frequency of pollinators (Gómez et al., 2010; Fernández et al., 2012). Our results indicated a negative relation between pollinator visitation frequency and the  $PL_C$  index in the manipulations. In addition, we also found preferably visited some studied patches. An explanation is that these patches have the higher pollen and nectar. This likely explains the difference in pollen limitation among the studied patches.

#### 4.2 Pollen Limitation and Resource Reallocation on Reproduction

Recent reviews demonstrate the outcomes of pollen-supplementation experiments may give misleading results because plants can reallocate resources among flowers during the pollination process (Knight et al., 2006; Gómez et al., 2010; Fernández et al., 2012). In addition, Baker et al. (2000) have also suggested single-flower manipulations potentially bias estimates of pollen limitation due to reallocate resources. To avoid confounding results related to resource reallocation, we have submitted the single-flower and whole-plant individuals to control or experimental treatments (Ryan & David, 2013). If plants differentially shunt resources to supplemented flowers at the expense of un-manipulated flowers (C treatment), the whole-plant individuals is more likely to be the higher seed set than the single-flower individuals because of the outcrossed offspring. However, we found the two controls of seed set were similar and were no differences between two manipulations, suggesting that resource reallocation, if existing, was weak.

#### 4.3 Self-Pollination and Reproductive Assurance

This self-pollination ability would provide reproductive assurance when pollinators fail. Our studies indicate *A. venetum* has a mixed mating system that involves both cross- and self-pollination. Recent reviews indicated the high success of self-pollination in the studied patches was an advantage in the pollination process, even during the period in which pollinators were scarce (Persy et al., 2012; Chen et al., 2016). If self-pollen is more likely to pollinate the ovary than cross-pollen, self-pollination may promote the pollination success, which is an automatic selection advantage (Jarne & Charlesworth, 1993).

These outcomes indicated the self-pollination ability would provide reproductive assurance when the pollinator fails to visit the flower. A recent meta-analysis shows that estimates of reproductive assurance are often biased when floral manipulations are performed on single-flower as compared to when whole-plant is manipulated (Knight et al., 2006; Ryan & David, 2013). When we compared estimates of reproductive assurance between single-flower and whole-plant manipulations, we found the level of manipulation did not significantly affect our estimates. In fact, if resource reallocation biases estimates of reproductive assurance, the seed set of emasculated treatment in the single-flower manipulations should be significant lower than that of the whole-plant manipulations because the latter is more likely to produce outcrossed offspring.

In this study, we found a negative relation between pollinator visitation frequency and the  $PL_C$  index in the manipulations, and insect pollination played a more important role in the outcrossing system. These outcomes indicated plant manipulations do not bias estimates of pollen limitation or reproductive assurance in *A. venetum*. In addition, more experimental field studies for resource reallocation are needed to understand in detail how common the phenomenon is and how it is related to life history and the costs of reproduction.

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