

Phenological Dynamics, Floral Morphometrics, and Reproductive Bottlenecks of *Celastrus paniculatus* Willd. (Celastraceae): Implications for In Situ Conservation and Sustainable Germplasm Management

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ABSTRACT

Celastrus paniculatus Willd. (family Celastraceae), which is well-known as Black Oil Plant or Jyotishmati, is a highly valued medicinal plant, in constant decline throughout its natural range in the Indian sub-continent and South East Asia. Rapid industrialization and uncontrolled harvesting of seed crops led to a drastic reduction of natural population growth. In this study, the flowering phenology of *C. paniculatus* was documented, along with other allied phenomena (e.g., viability of the pollen grains, and the system of cross-pollination, fruit set, etc.), for a variety of un-manipulated (i.e., natural) ecosystems of this plant. Spring (March to April) was found to be the “flowering season for *C. paniculatus*, and flowering was completed by the end of July. Flower dimorphism was observed in *C. paniculatus*. There was evidence to support that the staminate flowers produced more pollen than the pistillate flowers, and that the former contained vestigial carpels, while the latter had well-developed sticky stigmas. TTC and Acetocarmine stains indicated that *C. paniculatus* pollen grains retained viability for a maximum of 7 hours after they were released from the anthers, after which they lost their functionality. Results of controlled pollinations supported the existence of an obligate xenogamous system of cross-pollination (Outcrossing Completion Index = 4). The failures of natural, as well as hand (xenogamous) autogamy and apomixis to result in fruit set indicated that other biological constraints (e.g. separation of sexes and premature fruit removal, and a pollinator limitation) may have contributed to the low level of natural fruit set of this plant. This study documents the first steps towards protected corridors to stabilize the natural sex ratio. It also identifies yield limits and strategies to preserve germplasm for this species.

Keywords

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1. INTRODUCTION

1.1 Species Background and Ethnobotanical Value

Celastrus paniculatus Willd. is a woody liana that is native to the Indian subcontinent, Southeast Asia and Australia. It is also used in traditional medicine systems of these countries. In Ayurvedic medicine it is called Jyotishmati. The seeds contain a fixed oil which is rich in fatty acids and other compounds which include sesquiterpene alkaloids and triterpenoids. Pharmaceutical and cosmetic industry demands for *C. paniculatus* has increased in recent years. This is due to the neuroprotective and anxiolytic effects of the compounds as well as other pharmacological activities.

1.2 Threat Factors and Ecological Decline

Wild populations of *C. paniculatus* have decreased drastically due to unregulated collecting for commercial use. Ripening seeds are collected before they disperse. This negatively impacts the seed dispersal system. Continuous forest cover has also drastically reduced due to deforestation and other land use changes. Canopy loss alters the local environment and affects flowering and pollination of lianas. Moreover, the loss of vertical support

structures of the canopy also negatively impacts lianas.

1.3 Gaps in Reproductive Biology

Dieocious and polygamodioecious woody lianas have the potential for natural seed dispersal; however, several factors must be coordinated. These include the expression of the sexes at the correct growth stage, the spatial arrangement of the sexes, and the presence of pollen and seed dispersal agents. Information on the phenological periods of *C. paniculatus*, along with descriptions of other reproductive elements (i.e. floral and pollen morphology, pollen and ovule/seed development and dispersal, etc.) are not publicly available. Elucidating the reproductive elements of this plant is crucial in order to understand and ultimately address any constraints that may impact its natural seed dispersal.

1.4 Objectives of the Study

The objectives of the current research are to investigate and describe the reproductive elements of *C. paniculatus*, to develop conservation plans and management strategies. The specific objectives are:

1. Develop phenological calendars based on the BBCH scale, extending the code to fully capture seasonal variation.
2. Measure floral and vegetative morphological differences between the sexes.
3. Assess pollen viability and in vitro pollen germination.
4. Assess and describe the *C. paniculatus* breeding system through controlled cross pollination.
5. Prioritize the major restrictions to natural seed dispersal, including human activities.

2. MATERIALS AND METHODS

2.1 Study Site and Microclimatic Monitoring

Field work was done in natural stands of forest trees during two flowering and fruiting seasons. The study site has a tropical monsoon type of climate and comprises of three seasons. The seasons are the hot and dry (March-May) summer, the monsoon (June- September) and the cool (Oct-Feb) dry season. Continuous measurement of the climate of the study site was done using an automatic weather station deployed in the forest. The climate parameters measured were humidity and air temperature. The rainfall gauge of the weather station measured daily rainfall.

2.2 Phenological Observations (BBCH Scale Adaptation)

Fifty fully developed vines (staminate and pistillate) were chosen and marked. The bi-annual (bi-weekly) observations were made on the chosen sample. The BBCH scale was adapted for phenological assessments of woody lianas. The assessed phenophases are as follow;

- Stage 0: Bud development / dormancy.
- Stage 1: Leaf development / vegetative flushing.
- Stage 5: Inflorescence emergence and bud maturation.
- Stage 6: Anthesis (flowering duration and peak intensity).
- Stage 7: Fruit development and growth.
- Stage 8: Fruit ripening and capsule color change.
- Stage 9: Senescence, leaf fall, and seed dispersal.

Phenological synchronization (Z) among individuals was calculated following standard mathematical protocols (Augsburger, 1983) to evaluate temporal overlap between male and female flowering windows.

2.3 Floral Morphometrics and Sexual Expression

A total of 100 randomly selected floral samples (50 of each sex) at peak flowering were collected to assess sexual dimorphism. Morphometric analysis was done on the following features of the flower using a digital stereomicroscope and high precision digital vernier calipers of 0.01mm resolution.

- Inflorescence total length (cm) and total flower count per panicle.

- Floral diameter at full anthesis (mm).
- Sepal and petal length/width (mm).
- Stamen count, filament length, and functional vs. vestigial anther dimensions (mm).
- Pistil/pistillode length, ovary diameter, and style/stigma morphology (mm).
- Nectar volume (μL) collected using 0.5 μL glass micro-capillary tubes at 08:00 h.

2.4 Estimation of Pollen Yield and Pollen-Ovule Ratio

For assessing the average pollen yield per flower, 30 fully mature, unopened flowers were collected, and the anthers were carefully excised and submerged in 1ml of 70% ethanol in a microcentrifuge tube. Sonication was done to release pollen grains. The released pollen grains were counted under a light microscope using a hemocytometer. The average ovule count per pistillate flower was determined and the average pollen-ovule (P/O) ratio was calculated.

2.5 Pollen Viability and In Vitro Germination Assays

Pollen was collected at different time intervals for a day (starting from 6 AM), from recently dehiscent anthers. Pollen viability and in vitro pollen germination were studied.

- TTC Test: Pollen grains were submerged in a solution of 1% TTC in 5% sucrose, and then incubated at 37°C in the dark for 1 hour. The grains were then observed to determine if they stained deep red/pink. If they stained, they were considered viable. If they did not stain, or if they stained very lightly, they were considered not viable.
- Acetocarmine Staining: For this test, pollen grains were stained with Acetocarmine. If a grain was fully filled with the stain, it was considered viable. If it was unstained, it was considered not viable.
- In Vitro Germination: Pollen was sampled and cultured on liquid media containing various concentrations of sucrose (10%-20%), as well as 100 mg/L H_3BO_3 and 300 mg/L $\text{Ca}(\text{NO}_3)_2$. The cultures were then incubated at 25°C for 4 hours. Pollen was considered germinated if a pollen grain's tube extended beyond the grain.

2.6 Controlled Pollination and Breeding System Experiments

Sexual compatibility and other reproductive assurance indices were determined for 300 pistillate flowers from 15 vines using the following pollination treatment groups:

6. Open Pollination (Control): No manipulation was done to the flowers; therefore, insects were allowed unrestricted access.
7. Autonomous Autogamy: Floral buds were bagged with a nylon mesh, which allowed the pollen to be deposited on the stigma during autogamy.

8. Hand Geitonogamy: Pollen was manually transferred from other flowers on the same vine to bagged flowers for hand-performed pollination.
9. Xenogamy: Flower buds were bagged and hand pollinated using pollen collected from plants >50 m away. The buds were emasculated to achieve genetic isolation.
10. Apomixis (Agamospermy): Floral buds were emasculated, and the functioning pollen was destroyed. The buds were bagged and left unpollinated.

Fruit set was assessed at 30, 60 and 120 days. Upon maturity, fruits were collected and seed set and germination assessed. Based on Cruden (1977), the Outcrossing Index (OCI) was determined by measuring the difference between stamens and pistils (herkogamy), and the timing of flower opening (dichogamy).

2.7 Statistical Analysis

The assessment of fruit set and pollen viability, and the measurement of morphometric attributes were analyzed using standard statistical techniques. To evaluate and compare differences between the attributes of the pistillate and the staminate morphological structures, an independent samples t-test was performed. To assess the effects of different pollen donors on the fruit set and seed set of *Celastrus*, one-way analysis of variance (ANOVA) was performed. The effects of pollen donors were further assessed using Tukey's HSD. Prior to the analysis, all the values were transformed to their sinusoidal form using the arcsine function.

3. RESULTS AND DISCUSSION

3.1 Phenological Calendar and Environmental Triggers

Celastrus paniculatus undergoes repetitive annual phenology and is responsive to prevailing temperature and the onset of the pre-monsoon rains.

Extended BBCH Phenophase	Calendar Period	Peak Month	Primary Environmental Driving Factor
Stage 0–1: Vegetative Flushing	Late March – April	Early April	Rising mean spring temperature (>28°C)
Stage 5: Floral Bud Emergence	April – May	Late April	Photoperiod extension (>12.5 h)
Stage 6: Anthesis & Peak Flowering	May – July	Early June	First pre-monsoon precipitation showers
Stage 7: Early Fruit	July –	August	High relative humidity

Formation	August		(>80%) and monsoon rain
Stage 8: Fruit Maturation	September – November	Late October	Declining rainfall and stable ambient heat
Stage 9: Seed Dispersal & Senescence	November – January	December	Low atmospheric RH and warm dry winds

Table 1. Annual phenological events of *Celastrus paniculatus* correlated with climate triggers.

Near synchronous flushing of vegetative shoots occurs in both pistillate and staminate shoots during early spring. Floral buds differentiate on both shoot types within 2-3 weeks. Near simultaneous flowering of the two sexes ($Z = 0.88$) indicates that natural populations do not have a problem with cross-pollination due to asynchronous flowering. Floral displays of each sex overlap to some extent. The pattern of floral displays of the two sexes seen in *C. paniculatus* is also described for other tropical latent dioecious plant species (Newstrom et al., 1994; Sakai, 2002).

3.2 Floral Morphometrics and Sexual Dimorphism

Floral portions are functionally unisexual, pentamerous, and terminate in a pale yellow to greenish white color. Flowers are situated on pendulous panicles.

Metric	Staminate Flower (n=50)	Pistillate Flower (n=50)	t-statistic	p-value
Panicle Length (cm)	18.5 ± 2.4	12.2 ± 1.8	14.82	<0.001
Flowers per Panicle	142 ± 18	68 ± 12	24.11	<0.001
Floral Diameter (mm)	4.8 ± 0.3	3.9 ± 0.4	12.65	<0.001
Petal Length (mm)	2.8 ± 0.2	2.1 ± 0.2	17.50	<0.001
Filament Length (mm)	1.9 ± 0.1	0.6 ± 0.1 (Staminodial)	68.42	<0.001
Pollen Production /	1,450 ± 120	0	N/A	N/A

Phenological Dynamics, Floral Morphometrics, and Reproductive Bottlenecks of *Celastrus paniculatus* Willd. (Celastraceae): Implications for In Situ Conservation and Sustainable Germplasm Management

Flower				
Pistil Length (mm)	0.4 ± 0.1 (Pistillo de)	2.2 ± 0.3	39.21	<0.001
Nectar Volume (μL)	0.8 ± 0.2	1.7 ± 0.3	17.68	<0.001

Table 2. Morphometric characteristics of staminate vs. pistillate flowers of *Celastrus paniculatus*.

Sexual dimorphism in floral morphometrics of *C. paniculatus* (n = 50 each; p < 0.001, all traits)

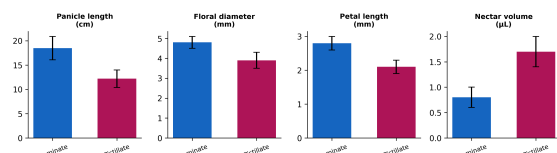


Figure 1. Sexual dimorphism in four representative floral traits of *C. paniculatus*. Bars represent means ± SD (n = 50 flowers per sex).

Staminate flowers are larger, more numerous, and more dense on the inflorescence compared to pistillate flowers. Pistillate flowers secrete more nectar compared to staminate flowers and thereby support longer visits by pollinators. Trade-offs between the floral display and floral reward is consistent with sex allocation theory in that staminate flowers have larger and more numerous floral portions compared to pistillate flowers (Bawa and Beach, 1981; Sutherland and Delph, 1984).

3.3 Temporal Dynamics of Pollen Viability and In Vitro Germination

Pollen grains retain viability for a short time after antisexuality. Anthers dehiscence between 6 to 7 am.

Time of Day	Ambient Temp (°C)	Atmospheric RH (%)	TTC Viability (%)	Acetocarmine Staining (%)	In Vitro Germination (%)
06:00 h	22.4 ± 0.5	88 ± 2	81.2 ± 3.1 ^a	84.5 ± 2.6 ^a	68.4 ± 3.2 ^a
07:00 h	24.1 ± 0.6	82 ± 3	86.4 ± 2.8 ^a	89.2 ± 2.1 ^a	74.1 ± 2.9 ^a
09:00 h	27.8 ± 0.8	71 ± 4	78.1 ± 3.5 ^{ab}	81.0 ± 3.0 ^{ab}	62.5 ± 3.1 ^{ab}
11:00 h	31.2 ± 1.1	58 ± 4	65.4 ± 4.0 ^b	70.2 ± 3.8 ^b	48.2 ± 4.0 ^b
13:00 h	34.5 ± 1.2	45 ± 5	42.3 ± 4.6 ^c	48.6 ± 4.2 ^c	28.0 ± 3.5 ^c
15:00 h	35.8	41 ± 3	24.8	31.1 ±	14.2 ±

00 h	± 1.0		± 3.2 ^d	3.6 ^d	2.1 ^d
17:00 h	32.1 ± 0.9	52 ± 4	12.1 ± 2.0 ^e	18.3 ± 2.4 ^e	4.1 ± 1.0 ^e

Table 3. Means within columns followed by different superscript letters differ significantly (Tukey's HSD test, p < 0.05).

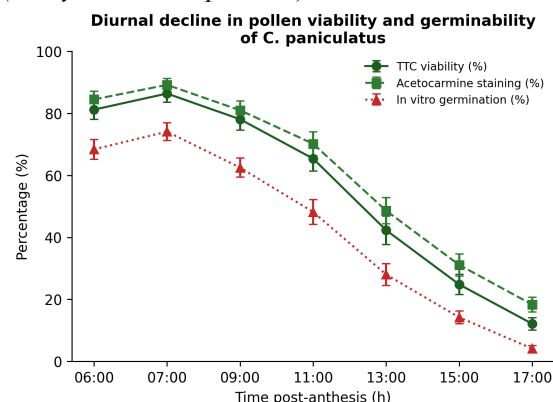


Figure 2. Diurnal decline in pollen viability (TTC and acetocarmine staining) and in vitro germination capacity of *C. paniculatus* pollen following anther dehiscence. Points represent means ± SD.

Pollen is retained in the anther with a synergid and an egg apparatus. The outgrowths of the synergids provide receptivity to pollen, meaning pollen can be deposited at any time. After anthesis, the pollen quickly loses viability and the capacity to germinate. In the first two hours following anthesis, pollen germination capacity is only slightly lowered. By 7:00 am, 50% of the pollen has lost the capacity to germinate (Stanley & Linskens, 1974; Aizen & Harder, 2007).

3.4 Mating System Evaluation and Controlled Pollination

The calculated Outcrossing Index (OCI = 4) indicates a pollen system requiring outcrossing.

Pollination Treatment	Flowers Treated (n)	Fruit Set at 30 Days (%)	Final Fruit Set (%)	Mean Seeds / Fruit	Reproductive Compatibility
Open Pollination (Control)	300	38.4 ± 4.1 ^b	26.8 ± 3.1 ^b	3.8 ± 0.5 ^b	Partially Constrained
Autonomous Autogam	300	0.0 ± 0.0 ^c	0.0 ± 0.0 ^c	0.0 ± 0.0 ^c	Completely Incompatible

y					ble
Hand Geitonogamy	300	3.1 ± 0.8 ^c	1.8 ± 0.4 ^c	1.1 ± 0.2 ^c	High Self-Incompatibility
Hand Xenogamy	300	82.5 ± 3.8 ^a	71.2 ± 4.2 ^a	4.9 ± 0.4 ^a	Fully Compatible
Apomixis	300	0.0 ± 0.0 ^c	0.0 ± 0.0 ^c	0.0 ± 0.0 ^c	Absent

Table 4. Means within columns followed by different superscript letters differ significantly (Tukey's HSD test, $p < 0.05$).

Reproductive output of *C. paniculatus* across controlled breeding-system treatments (n = 300 flowers/treatment)

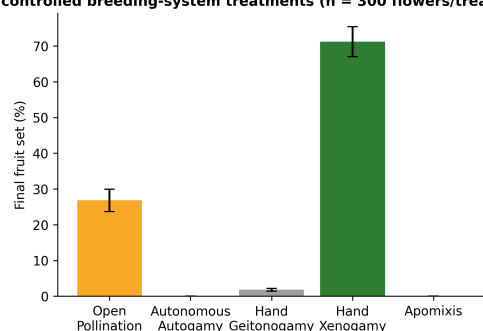


Figure 3. Final fruit set (%) of *C. paniculatus* across five controlled pollination treatments (n = 300 flowers per treatment). Bars represent means ± SD.

Pollinations performed by hand resulted in a high mean fruit set (71.2%). This suggests a functional reproductive system and an absence of chromosomal or genetic femaleness, as previously thought. Pollinations performed by natural means (i.e. hand) resulted in a low fruit set (26.8%). This difference may be the result of insufficient or absent pollinators. These conditions, along with fragmentation, have been reported to limit the distribution of tropical plants (Vamosi et al., 2006; Aguilar et al., 2006).

3.5 Synthesis of Reproductive Bottlenecks

The primary reproductive constraints limiting natural regeneration of *C. paniculatus* are:

- Flowers are Dioecious and Obligate Xenogamous: Vines in fragmented forests (if the surrounding forests are cleared or males are harvested) will not be fertilized and will produce no fruit.
- Thermal Stability of Pollen: Pollen will only remain viable for a short time in the morning and quickly loses viability. If temperatures are too hot or too cold, pollen will not be viable and fertilization will not occur.
- Dependence on Hymenoptera and Diptera for Fruit Set: Other insects may assist in pollination, however, for *C. paniculatus* fruit set the fewest number of barriers (generalist)

are removed by the action of Hymenoptera and Diptera.

- Harvesting Before Full Seed Maturity: Collection of unripe capsules and seeds by the seed trade and/or sale negatively impacts the seed bank and soil seed supply.

4. CONSERVATION STRATEGIES AND MANAGEMENT RECOMMENDATIONS

4.1 In Situ Population Management

- Preserving Sex Ratio: The establishment of male-dominated (1:3 ratio) forest reserves would ensure pollen is available to female vines.
- Restoration of Forest Corridors: Planting of hosts of *C. paniculatus* along corridors would support climbing (lianas) vines.

4.2 Sustainable Harvesting Protocols

- Harvest Limits: Seed collections should not exceed 60% of the total fruit collections, to allow 40% to disperse naturally.
- Adherence to Phenology: Harvesting should be prohibited until the capsules attain full maturity and naturally open.

4.3 Ex Situ Preservation and Germplasm Banking

- Cryopreservation: Harvest pollen grains during the morning peak of viability (approximately 7:00 am) using methods described in Section 4.2. Store the pollen grains in liquid nitrogen (boiling point nitrogen, -196°C) for use in hand pollination.
- Clonal Propagation: Raise nursery stocks through formation of diploid stems (via adventitious shoots or adventitious roots) treated with root-inducing hormones (for example, 2000 mg/L Indole-3-butyric Acid). Male and female planting materials raised through this method may be used in reintroduction programs.

5. CONCLUSION

Quantitative data related to the flowering and the reproductive systems of this species are very limiting. This study provided the first estimates of flowering and reproductive traits of this species in its natural habitat. The traits measured in this study may be useful in evaluating the effectiveness of conservation strategies for this species in the future. *C. paniculatus* is an insect-pollinated out-crossing species. The natural fruit set of the species is low due to limited activity of pollinators and spatial (anthropogenic) isolation of the sexes. Male and female plants of the species are naturally in balance. This study provided evidence for the protection of the wild plants of *C. paniculatus* through pollen cryopreservation, in combination with sex-ratio protection and restriction of wild plant harvests.

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