

# Phenology and reproductive biology of *Plukenetia corniculata* Sm., a traditional wild vegetable of Southeast Asia

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## Research Article

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# Abstract

## Background

*Plukenetia corniculata* Sm., a member of the family Euphorbiaceae, is a traditional leafy vegetable in Southeast Asia. Its young shoots, leaves, and young fruits are consumed as vegetables, and its mature seeds can be eaten as nuts. Although *P. corniculata* is not included in the list of threatened species, habitat destruction has affected its survivability in some areas. To enhance the conservation and exploitation of *P. corniculata* germplasm, more knowledge is necessary to elucidate its flowering characteristics and reproductive biology.

## Results

The results showed that the inflorescence buds of *P. corniculata* began to appear at approximately 98.72 days after sowing and fruits matured after another approximately 55.33 days. Pistillate flowers always blossomed approximately 5.07 days earlier than that of the first staminate flowers on the same inflorescence. On average, the anthesis of a single staminate flower lasted approximately 9.44 hours, pistillate flower 10.50 days, and the whole inflorescence 26.57 days. The pollen viability reached a maximum of 73.58% at the fourth hour after the staminate flower blooming, and the stigma receptivity usually lasted for approximately 9 days and reached a peak on the sixth day after stigma dehiscence. The pollen-ovule ratio (P/O) and outcrossing index (OCI) values of *P. corniculata* were 1607.09 and 3, respectively, suggesting its breeding system was classified as facultative xenogamy and self-compatible. Artificial pollination experiments furtherly confirmed that both self- and cross-pollination were compatible in *P. corniculata*. Additionally, seeds of *P. corniculata* had 70.67% kernel percentage and the oil content of kernels reached 58.65%.

## Conclusions

This study provided comprehensive data on flowering and fruiting phenology, reproductive characteristics, and breeding system of *P. corniculata*. The optimal time for emasculation, pollen collection, and artificial pollination were also determined in *P. corniculata*. High kernel percentage and oil content of *P. corniculata* seeds are worthy of further study and exploitation. The work lays a foundation for conservation and further breeding strategy of *P. corniculata*.

## Background

*Plukenetia* L. is a small pantropical genus in the family Euphorbiaceae and comprises approximately 23 species [1]. These species are distributed in the tropical regions of Central and South America, Africa, Madagascar, and Southeast Asia [2, 3]. Some *Plukenetia* species have been traditionally cultivated for edible seeds, seed oil, leafy vegetables, or medicinal properties in their distribution regions, such as

*Plukenetia volubilis*, the economically most important species of the genus, *Plukenetia carolis-vegae*, *Plukenetia huayllabambana*, *Plukenetia polyadenia*, and *Plukenetia conophora*. Their large edible seeds contain very high amounts of the polyunsaturated, essential fatty acids linoleic acid and  $\alpha$ -linolenic acid, and protein [1]. Moreover, the young shoots and leaves of *P. volubilis* and *Plukenetia corniculata* are used as vegetables in some areas [4, 5]. These *Plukenetia* species are potentially valuable germplasm resources for domestication and genetic improvement.

*P. corniculata* is a monoecious vine or slender liana and the only known species of *Plukenetia* distributed in Asia. It is widespread but uncommon throughout Southeast and South Asia, and has been reported in Northeast (Assam) and Southeast (Andhra Pradesh) India, Myanmar, Thailand, the Philippines, Malaysia, and Indonesia [3, 6]. The species is most densely distributed on the island of Sumatra and less so on Borneo and appears to be scattered and sparse outside this distribution area [3]. The reason might be due to its vine habit and lacking support plants in these areas. It previously had been reported to occur in open grounds but was absent from natural habitats later in Singapore [7]. Pradheep et al. (2015) could not visit the wild habitats of the plant in field trips to Assam and Nagaland of India, where it is cultivated as vegetable in a large way or at the homestead level to meet the local market needs of the nearby town. The growth of the plant may be strict with the environmental conditions, and habitat change may seriously affect the survival of the species. Although not included in the list of threatened species, it may be vulnerable in some areas of its habitats. On the other hand, it has great exploitation and utilization potential. The young shoots, leaves, and young fruits of the species are consumed as vegetables in some area [4]. Its leaves have a sweet taste accounting for its Assam local name 'meetha patta' (meetha means sweet, patta means leaf), while its mature seeds are eaten as nuts, which taste similar to peanuts [3, 4]. Now it is cultivated for leafy vegetable in Sarawak of Malaysia, Sumatra of Indonesia, and Assam and Nagaland of India [3, 4]. The study of the reproductive biology of a plant species may not only facilitate to reveal the underlying mechanisms for its survival strategies, but also provide scientific guidance for further conservation and exploitation of the species.

Studies on the reproductive biology of flowering plants include phenology, floral characteristics, pollination biology, and breeding system [8]. Flowering phenology is an important life-history trait because the timing of flowering and the schedule of reproductive expenditures across time can strongly influence individual seed production and ultimately fitness [9]. Pollen viability and stigma receptivity are also particularly important for successful pollination and fruit and/or seed set in flowering plants [10] and can be used to determine the optimum time to perform artificial pollination [11]. The breeding system plays an important role in the generation of variation and hence in the evolution of a species [12] and is divided into three categories: selfing, mixed mating, and outcrossing [13]. The pollen-ovule ratio (P/O) is a useful indicator of breeding systems in plants and is a methodology validated by several studies [14–16]. In addition, outcrossing index (OCI) based on floral morphological characteristics may also be used to estimate the breeding systems in plants [17]. In addition, controlled artificial-pollination experiments can further determine the breeding system [14].

Most of the research works have focused on the seed nutrients, oil extraction and composition nutritional composition and product development of the seeds in *Plukenetia* [18–24] and to some extent on genetic diversity, phylogeny, transcriptome, and high-yield cultivation [1, 3, 25–28]. However, the knowledge regarding phenological characteristics and reproductive biology of *Plukenetia* is rudimentary. Thus, we investigated the phenology, floral characteristics, and breeding system of *P. corniculata* to determine the optimal time and the right stage of flower buds for performing emasculation and pollination, which could promote the success rate of artificial hybridization with its closely related species and design strategies for conservation, cultivation, and sustainable exploitation of *P. corniculata*.

## Results

### Flowering and fruiting phenology

*P. corniculata* is a woody liana species that is perennial and evergreen. The duration of the vegetative growth stage of *P. corniculata* was approximately 98.72 days (Table 1). It is monoecious with separate pistillate and staminate flowers on the same inflorescence. According to the development of pistillate and staminate flowers, we divided the inflorescence development of *P. corniculata* into four stages: inflorescence bud stage ( ), initial blossoming stage of pistillate flower ( ), initial blooming together stage of the first staminate flower on the inflorescence ( ), and synchronous blooming stage of several staminate flowers on the inflorescence ( ) (Fig. 1). Although the pistillate and staminate flowers were produced at different positions of the inflorescence axis, it was not easy to distinguish them at stage , because each flower bud was completely wrapped by the bracts (Fig. 1A). Then inflorescence morphology varied significantly with its development. Stigmas dehiscence of the pistillate flower represented its blossoming, and pistillate flowers began to blossom at approximately 8.33 days after appearance of inflorescence buds (Fig. 1B), and the blossoming duration of a single pistillate flower was approximately 10.50 days (Table 1). Approximately 5.07 days after pistillate flower blossomed, the first staminate flower located at the base bloomed firstly, and the blooming duration of a single staminate flower was approximately 9.44 hours (Fig. 1C, Table 1). Staminate flowers bloomed gradually from the base to the apex of the inflorescence, and the number of bloomed staminate flowers per inflorescence was approximately one to three in a day (Fig. 1D). There was only one flower (rarely 2) on each node, one pistillate flower (rarely 2) on the basal node, and numerous staminate flowers were borne on the upper part of the inflorescence axis (Fig. 1B, C, D). The flowering period of a single inflorescence was approximately 26.57 days (Table 1). Some young fruits have developed and swelled when the staminate flowers began to bloom on the inflorescence (Fig. 1C, D). It took approximately 50.33 days from pistillate flower blossoming to fruit maturity. *P. corniculata* can produce inflorescences and fruits all year around in Xishuangbanna, with a considerable decrease during the dry season (November–April). Most data of this study was collected in the rainy season (June–October).

**Table 1** Flowering and fruiting phenology of *P. corniculata*

| Development stage                                                       | Duration time (days) |
|-------------------------------------------------------------------------|----------------------|
| Vegetative growth stage                                                 | 98.72±8.36           |
| From inflorescence initiation to the first pistillate flower blossoming | 8.33±2.25            |
| From inflorescence initiation to the first staminate flower blooming    | 13.40±3.20           |
| A single pistillate flower from blossoming to wilting                   | 10.50±2.07           |
| A single staminate flower from blooming to wilting                      | 9.44±2.13*           |
| Anthesis of a single inflorescence                                      | 26.57±3.95           |
| From pistillate flower blossoming to fruit maturity                     | 50.33±6.22           |

Note: \* hours.

### Floral characteristics

The inflorescences of *P. corniculata* were borne on the opposite side of leaves, and the average length was approximately 3.93 cm (Table 2). Both of staminate and pistillate flowers had four sepals, and their petals were lacking. The color of sepals turning pale green and opening represented the blooming of staminate flowers. Stigmas dehiscence meant the blossoming of pistillate flowers. The diameters of open pistillate and staminate flowers were approximately 3.21 and 3.22 mm, respectively (Table 2). Stigmas were approximately 1.93 mm in diameter and styles were short and approximately only 1.22 mm in length. Each pistillate flower contained 4 (rarely 5) winged ovaries, and each ovary had one oblate ovule. There were approximately 4.01 ovules and 12.36 stamens per pistillate and staminate flower, respectively. The detailed floral quantitative characteristics are shown in Table 2. Four stigmas of young pistillate flowers were fused into a thick disc with a cross-shaped surface (Fig. 2A). With the development of pistillate flowers, their stigmas began to dehisce and were emerald green (Fig. 2B). The dehiscence of stigmas reached its maximum level and many projections sprouted on the stigmas on the fourth-sixth days (Fig. 2C). Stigmas became yellowish green and senescent on the tenth days of dehiscence. (Fig. 2D).

**Table 2** Floral characteristics of *P. corniculata*

| Floral characteristics             | Mean $\pm$ SD    |
|------------------------------------|------------------|
| Inflorescence length (cm)          | 3.93 $\pm$ 0.64  |
| Pistillate flower diameter (mm)    | 3.21 $\pm$ 0.11  |
| Staminate flower diameter (mm)     | 3.22 $\pm$ 0.31  |
| Stigma diameter (mm)               | 1.93 $\pm$ 0.22  |
| Style length (mm)                  | 1.22 $\pm$ 0.23  |
| Ovule number per pistillate flower | 4.01 $\pm$ 0.11  |
| Stamen number per staminate flower | 12.36 $\pm$ 3.20 |

### Pollen viability and stigma receptivity

Staminate flowers of *P. corniculata* often bloomed at 8:00-9:00 am and wilted at 4:00-5:00 pm. The pollen viability exhibited a trend of first increasing and then decreasing with the flower blooming (Fig. 3), in which pollen viability was low (28.81  $\pm$  4.86%) at the beginning of flower blooming, then increased rapidly and reached the highest value (73.58  $\pm$  3.62%) at 4 hours after flower bloomed, and maintained a certain viability (65.09  $\pm$  5.06%) till 6 hours, and then decreased dramatically with flower blooming time (Fig. 3).

The hydrogen peroxide test detected that the stigma of *P. corniculata* had no activity reaction when it dehiscid slightly on the first day (Table 3). The stigma reaction gradually increased with dehiscing and reached a high level on the fourth-sixth day. Then, the reaction began to decline and remained a certain level till the ninth day (Table 3). Further artificial pollination experiments also obtained the similar results. On the first day of stigma dehiscence, few pistillate flowers could develop into fruits by artificial pollination. Moreover, the fruit setting rate was extremely low in the pistillate flowers pollinated on the second and third days after the stigma dehiscence. Then, the fruit setting rate began to increase rapidly with stigma dehiscence and reached the highest level (66.67%) of pollination on the sixth day. The fruit setting rate began to decrease rapidly with stigma senescence and stigmas lost their vitality approximately on the tenth day (Table 3).

**Table 3** Stigma receptivity and fruit setting rate of *P. corniculata* at different days after dehiscence

| Days after stigma dehiscence | Degrees of stigma reaction | Fruit setting rate(%)   |
|------------------------------|----------------------------|-------------------------|
| 1                            | –                          | 0                       |
| 2                            | +                          | 16.82±3.36 <sup>d</sup> |
| 3                            | ++                         | 30.91±4.93 <sup>c</sup> |
| 4                            | +++                        | 46.83±4.48 <sup>b</sup> |
| 5                            | +++                        | 50.16±5.87 <sup>b</sup> |
| 6                            | +++                        | 66.67±6.91 <sup>a</sup> |
| 7                            | +++                        | 27.62±4.46 <sup>c</sup> |
| 8                            | +                          | 13.12±3.71 <sup>d</sup> |
| 9                            | +                          | 11.08±4.35 <sup>d</sup> |
| 10                           | –                          | 5.92±3.95 <sup>e</sup>  |

Note: –, no reaction; +, weak positive reaction; ++, strong positive reaction; +++, very strong positive reaction. Different superscript letters indicate significant differences at  $P < 0.05$ .

#### Pollen-ovule ratio (P/O) and outcrossing index (OCI)

The mean numbers of pollen grains per staminate flower of *P. corniculata* was approximately 6416.67. Most pistillate flowers had 4 locules (occasionally 5) and per locule had one ovule. The mean number of ovules per pistillate flower was 4.01. Then, P/O of *P. corniculata* was approximately 1607.09 (Table 4). According to the standard of Cruden (1977), the breeding system of *P. corniculata* belonged to facultative xenogamy.

The average diameters of opening pistillate and staminate flowers were 3.21 mm and 3.22 mm, respectively (Table 2), which were between 2 mm and 6 mm, so this was scored as '2' following the standard of Dafni (1992). Pistillate flowers matured first (Table 1), so a score of '0' was recorded. Staminate flowers were always spatially higher than pistillate flowers, and their spatial separation was hence recorded as a score of '1'. According to Dafni (1992) standard, the OCI of *P. corniculata* was 3 by calculating the sum of the above three scores. Therefore, the breeding system of *P. corniculata* was self-compatible and sometimes requiring pollinators.

#### Table 4 Estimation of breeding systems in *P. corniculata*

| Number of pollen grains per staminate flower | Number of ovules per pistillate flower | P/O     | Breeding systems     |
|----------------------------------------------|----------------------------------------|---------|----------------------|
| 6444.45±993.81                               | 4.01±0.11                              | 1607.09 | facultative xenogamy |

## Artificial pollinations

The fruit set exhibited obvious differences among various pollination treatments. The control, namely natural pollination, had a fruit setting rate of 64.18% after 4 weeks of pollination (Table 5). The fruit setting rate of the emasculated and non-bagged treatments was 61.33%, which was similar to that of the natural pollination. The bagged and non-emasculated treatments had a fruit setting rate of 49.62%, whereas the emasculated and bagged treatments showed no fruit production. The fruit setting rates of both the geitonogamy and xenogamy treatments were relatively high, reaching 73.71% and 76.29%, respectively (Table 5). These results indicated that pistillate flowers of *P. corniculata* were easily pollinated by pollen grains from the same or other individuals.

**Table 5** Fruit set under different pollination treatments of *P. corniculata*

| Treatment                            | Fruit setting rate[%]   |
|--------------------------------------|-------------------------|
| Natural pollination (control)        | 64.18±3.83 <sup>b</sup> |
| Emasculated and not bagged           | 61.33±4.76 <sup>b</sup> |
| Bagged and not emasculated           | 49.62±5.51 <sup>c</sup> |
| Emasculated and bagged               | 0                       |
| Emasculated, bagged, and geitonogamy | 73.71±5.48 <sup>a</sup> |
| Emasculated, bagged, and xenogamy    | 76.29±6.65 <sup>a</sup> |

Different superscript letters indicate significant differences at  $P < 0.05$ .

## Fruit and seed characteristics

According to the fruit development stage, seven stages were classified as follows: unpollinated ovary (UPO), beginning to develop stage (3 days after pollination, 3 DAP), early development and growth stage (6 DAP), rapid swelling stage (10 DAP), rapid swelling stage (20 DAP), green adult stage (30 DAP), and ripening stage (50 DAP) (Fig. 4A). Fruit wings of *P. corniculata* obviously elongated at 3 DAP, as well as portended the fruit began to develop. Fruits entered the stage of rapid growth from 10 DAP and reached the size of adult fruits at 30 DAP (Fig. 4A). Fruits ripened at approximately 50 DAP, and fruit shells spontaneously burst to disperse seeds. The size of adult fruits was average 1.97cm, 1.72 cm, and 1.14 cm in length, width, and thickness, respectively (Table 6). Fruits of *P. corniculata* were primarily 4-lobed capsules, and each carpel lobe developed a strap-shaped wing whose length was approximately

1.11 cm (Table 6). There were also a few fruits of the 4-lobed capsules with one ovule abortion or the 5-lobed capsules (Fig. 4B). Seeds of *P. corniculata* were broadly lenticular, approximately 1.01 cm, 0.81 cm, and 0.62 cm in length, width, and thickness, respectively, and had medium-brown and persistent testa (Fig. 4C, Table 6). The thousand-seed weight was approximately 164.90 g. The seeds had a high percentage of kernels which reached approximately 70.67%. The kernel oil content was approximately 58.65% (Table 6).

**Table 6** Fruit and seed characteristics of *P. corniculata*

| Characteristics          | Mean ± SD    |
|--------------------------|--------------|
| Fruit length (cm)        | 1.97±0.21    |
| Fruit width (cm)         | 1.72±0.18    |
| Fruit thickness (cm)     | 1.14±0.12    |
| Fruit wing length (cm)   | 1.11±0.08    |
| Seed length (cm)         | 1.01±0.03    |
| Seed width (cm)          | 0.81±0.03    |
| Seed thickness (cm)      | 0.62±0.02    |
| Thousand-seed weight (g) | 164.90±10.43 |
| Kernel percent (%)       | 70.67±1.31   |
| Kernel oil content (%)   | 58.65±1.64   |

## Discussion

Knowledge of flowering and fruiting phenology is important in understanding the adaptation of a plant to its environment and devising an efficient conservation or breeding strategy. Flowering and fruiting phenology have been studied in only a few *Plukenetia* species. The initial flowering of *P. volubilis* usually occurs approximately 5 months after transplanting or sowing [29, 30] and *P. conophora* commences flowering between one and a half to two years after transplanting [31, 32]. Fruit maturity of *P. volubilis* and *P. conophora* usually takes approximately 3–5 months [30, 33] and 4–6 months [34], respectively. *P. corniculata* starts flowering one and a half years after sowing in Nagaland, India [4]; however, the inflorescence buds of *P. corniculata* began to appear approximately 98.72 days and flowered 107.05 days (less than 4 months) after transplanting to the field in XTBG, Mengla, Yunnan, China (Table 1). The difference in flowering time might result from different growing environments and/or different germplasms. Fruits of *P. corniculata* matured approximately 50 days (less than 2 months) after pistillate flower blossoming. These results showed that *P. corniculata* had a short breeding cycle and could be used to accelerate interspecific cross-breeding of *Plukenetia*.

Similar to the majority of species of *Plukenetia*, *P. corniculata* is monoecious with bisexual inflorescences [3]. Only one pistillate flower (rarely 2) was borne on the basal node, and numerous staminate flowers were borne on the upper part of the inflorescence axis (Fig. 1). The inflorescence architecture of *P. corniculata* might be beneficial to selfing because pistillate flowers were easily pollinated by pollen from staminate flowers on the same inflorescence. In addition, the style length of *Plukenetia* varies considerably; for example, styles of *P. corniculata* were short and only approximately 1.22 mm in length (Table 2), while the style length of *P. conophora* and *P. volubilis* is approximately 4–8 mm and 15–30 mm, respectively [3, 35]. The difference in style length might be related to the pollination habit of these *Plukenetia* species. Pistillate flowers blossomed approximately 5.07 days earlier than that of the first staminate flower on the same inflorescence in *P. corniculata*. The duration of a single staminate flower blooming was only approximately 9.44 hours, and the pollen viability remained high within 4–5 hours after staminate flower bloomed (Fig. 3). Stigma receptivity could sustain for approximately 10 days and reached a high level on the fourth to sixth day after stigma dehiscence (Table 1, Fig. 3B). These results indicated that there was a certain period of overlap between stigma receptivity and pollen viability, and the peak receptive stage of stigma almost met the staminate flower blooming on the same inflorescence. These characteristics allow pistillate flowers to be pollinated within the same inflorescence while also having enough time to be pollinated by pollen grains from other inflorescences of the same plant or other individuals.

According to the values of P/O and OCl, the breeding system of *P. corniculata* was deduced to belong to facultative xenogamy, self-compatible, and sometimes requiring pollinators. Artificial pollination experiments further confirmed the results (Table 5). The fruit setting rate of the emasculation and non-bagged treatments was almost the same as that of the natural pollination of *P. corniculata*, which indicated that cross-pollination by pollen grains from other inflorescences might be the primary pollination strategy for *P. corniculata*. The bagged and non-emasculated treatments defined *P. corniculata* as a self-compatible species because the fruit setting rate reached 49.62% in autonomous self-pollination experiment. There was no fruit production in the inflorescences of the emasculated and bagged treatments, indicating that apomixis did not occur in *P. corniculata*. In addition, the fruit sets in both the geitonogamy and xenogamy treatments reached 73.71% and 76.29%, respectively (Table 5), which further indicated that both self- and cross-pollination were compatible in *P. corniculata*. These results will lay a foundation for artificial pollination in identifying the right stage to practice emasculation, selfing, and crossing for use in genetic improvement programs and help provide a reference for the study of breeding systems in other *Plukenetia* species.

The seeds of *Plukenetia* are rich in oil and protein and can be used to extract oil or eaten directly by local people [3, 23]. Mature seeds of *P. corniculata* can also be edible and have a taste similar to that of peanuts [3]. According to the size categories by Cardinal-McTeague et al., *P. corniculata* seed belongs to M (medium, 123–272 mm<sup>3</sup>) in *Plukenetia* [1]. The sizes of the seeds produced in XTBG were approximately 1.01 cm, 0.81 cm, and 0.62 cm in length, width, and thickness, respectively (Table 5), which were similar to that of the seeds produced in other areas [3]. The thousand-seed weight of *P.*

*corniculata* was approximately 164.90 g, which is obviously lower than that of commonly consumed species, such as *P. volubilis* (880–1330 g) [36] and *P. conophora* (9500 g) [37]. However, the seeds had 70.67% kernel percentage and the kernels contained 58.65% oil (Table 5). The kernel oil content was obviously higher than that of *P. volubilis* (38.15–48.81%) [38] and *P. conophora* (48–52%) [39]. Therefore, seeds still need to be further exploited besides young shoots, leaves, and young fruits of *P. corniculata*. The characteristics of the high oil content in kernels of *P. corniculata* can also be used in the genetic improvement of other species in *Plukenetia*.

## Conclusions

This study demonstrated that *P. corniculata* had a short breeding cycle and only needed approximately 5 months from sowing to fruit maturity. Stigma receptivity of *P. corniculata* reached its peak on the sixth day after stigma dehiscence. Pollen viability maintained at a high level for 4–5 hours and reached a maximum at the fourth hour after the staminate flower blooming. Therefore, collecting the pollen at the fourth hour of staminate floral anthesis and pollinating pollen on the stigma on the sixth day after stigma dehiscence can improve the pollination rate of *P. corniculata*. The P/O and OCI values suggested that the breeding system of *P. corniculata* was classified as facultative xenogamy, self-compatible, and sometimes requiring pollinators, which were furtherly confirmed by artificial pollination. Seeds of *P. corniculata* had 70.67% kernel percentage and the kernels contained 58.65% oil, which are worthy of further study and exploitation. To the best of our knowledge, this is the first study to provide comprehensive data on flowering and fruiting phenology, floral characteristics, and breeding system of *P. corniculata*. It not only reveals the reproductive characteristics and the optimal time for emasculating and pollinating *P. corniculata* but also provides a certain reference value for researching on the breeding systems of other species in the genus.

## Materials And Methods

### Plant materials

*P. corniculata* seeds were collected from Niah (3°47' N, 113°46' E, 90 m a.s.l.), Sarawak, Malaysia, and introduced to Xishuangbanna Tropical Botanical Garden (XTBG) of the Chinese Academy of Sciences (21°54' N, 101°46' E, 580 m a.s.l.), located in Mengla County, Yunnan Province, China. The seeds were sown in polythene cups filled with sterilized soil consisted of humus, peat, and vermiculite in the ratio of 3:1:1, and then germinated in an incubator at 30°C, 14 h light/10 h darkness, and 70% relative humidity. The seedlings emerged at 10 to 15 days after sowing. Thirty days later, the young seedlings were transplanted in the field with a spacing of 1.5 m between plants within a row and 2 m between rows. Data on different aspects of reproductive biology in *P. corniculata* were collected from these plants.

### Phenological Observation Of Flowering And Fruiting

The flowering and fruiting phenology were recorded on 50 randomly selected plants from March 2019 to October 2021. Vegetative growth stage was recorded as the number of days from sowing to appearance of the first inflorescence bud. The number of individuals sprouting the first inflorescence bud was recorded daily, and the reproductive stage was identified when 50% of individuals sprouted inflorescence buds. Three inflorescence buds on each individual were randomly selected and tagged. The duration from inflorescence initiation to first flowering, the blossom duration of a single flower, and the blossom duration of a single inflorescence were counted on 30 inflorescences from different individuals, respectively. The stigma dehiscence of pistillate flowers were observed every day, and the staminate flowers were observed every 1 hour on the day of anthesis until they wilted. The flowering duration of a single inflorescence was recorded from the opening of the first flower to the wilting of the last flower on the inflorescence axis, and the observations were recorded daily during flowering in the afternoon. The duration of fruit development was recorded as the time from pistillate flowering to fruit maturity based on the observation of 100 tagged pistillate flowers every 2 days.

## Flower, Fruit, And Seed Characteristics

To examine the floral characteristics of *P. corniculata*, flower diameter, style length, stigma diameter, and ovule number of per pistillate flower were measured on 30 fully opened flowers randomly selected from different inflorescences of different individuals. In addition, stamen number per staminate flower was counted on 30 freshly opened staminate flowers randomly selected from different inflorescences. Fruit and seed characteristics such as length, width, and thickness were measured based on 60 fully developed green fruits and 100 mature seeds collected from 30 individuals, respectively. An electronic Vernier caliper was used to measure the above data.

## Pollen Viability And Stigma Receptivity Detections

The methyl thiazolyl tetrazolium (MTT) staining method [40] was used to measure the pollen viability of *P. corniculata*. One hundred staminate flowers were tagged one day before flowering. Ten flowers just opening were randomly collected in different individuals at about 8:00 am, and then another ten opening flowers were randomly collected once every 2 hours until the flowers wilted at about 6:00 pm. Pollens were obtained from 3–5 anthers randomly selected from each flower and were dusted on glass slides to which 1–2 drops of MTT solution were added. All slides were observed under an optical microscope (Leica DM 2500, Germany). Pollen grains appearing dark red were considered viable, whereas slightly stained or colorless pollen grains were considered nonviable. The percentage of pollen viability was evaluated as the ratio of the number of viable pollen grains to the total pollen grains in five randomly focused fields of the microscope. The measurements were repeated three times on different dates. To evaluate stigma receptivity, the hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) method was used as described previously [14]. One hundred pistillate flowers that were predicted to open in the following day were tagged from different plants, and ten pistillate flowers were randomly selected from different individuals from about 10:00–

11:00 am per day for ten days. Thereafter, a drop of 6% H<sub>2</sub>O<sub>2</sub> was deposited on the stigmas of selected pistillate flowers, and the number of bubbles that emerged from the stigma within 1 min was used as the index assessing stigma receptivity [41].

## Pollen-ovule Ratio (P/o) And Outcrossing Index (Oci) Analysis

To evaluate the reproductive system of *P. corniculata*, P/O was used in this study according to Cruden [42]. Thirty staminate and pistillate flower buds were randomly selected from 30 individuals. Six mature but unopened staminate flowers were collected and placed in a 1.5 ml centrifuge tube and air dried for 24 hours to open the anthers. Then, 1 ml suspension solution (70% ethanol and 0.5% Triton-X, v/v) was added to the centrifuge tube, and the tube was placed in a sonic bath for 5 min to release pollen grains. The tube was vortexed for 1 min to evenly distribute the pollens in the solution. Then, 20 µl of the pollen suspension was immediately dropped into a hemocytometer chamber, and the number of pollen grains was counted under a microscope (Leica DM2500, Germany). The procedure was repeated five times, and the mean number of pollen grains per staminate flower was calculated. The number of ovules per pistillate flower was counted under a stereomicroscope by dissecting the ovary. P/O was calculated by dividing the mean number of pollen grains per staminate flower by the mean number of ovules per ovary. Thirty inflorescences were randomly selected for the evaluation of OCI. The flower size was measured, and the opening time and the spatial position of staminate and pistillate flowers on the same inflorescence were recorded. The OCI value was assessed based on the above results according to Dafni's standards [17].

## Controlled Pollination Experiments

To further establish the pollination system of *P. corniculata*, 180 inflorescences with mature but unopened flowers were randomly selected from 30 individuals and divided into six groups (30 inflorescences in each group) for various pollination experiments. The following treatments were designed: 1) natural pollination (control): the inflorescences were exposed to pollinators to detect the ratio of fruit-setting in its natural conditions; 2) opening pollination by pollen from other inflorescences: the inflorescences were emasculated and not bagged to detect the contribution of foreign pollen; 3) spontaneous self-pollination: inflorescences were bagged with mesh nylon net and not emasculated to detect whether spontaneous self-pollination exists; 4) apomixis: the inflorescences were emasculated and bagged to detect whether apomixis exists; 5) artificial self-pollination: the inflorescences were emasculated and bagged, and their stigmas were artificially pollinated with pollen from staminate flowers of the same individual to detect self-compatibility; 6) artificial cross-pollination: the inflorescences were emasculated and bagged, and the pollen for artificial pollination was from different individuals of the same species to detect cross-compatibility. The suitable stage of staminate and pistillate flowers was chosen, and stamens or staminate flowers were collected with a tweezer and transferred onto stigmas twice per day at about 10:00–11:00 am for three days. The pistillate flowers were timely bagged again to avoid manipulation by

pollinators after artificial pollination. The fruit setting rate of each treatment was monitored 5–6 weeks after pollination.

## Data analysis

Data analyses were performed using Statistical Product and Service Solution software (version 16.0, SPSS Inc., Chicago, IL, USA), and the data are presented as the mean and standard deviation (SD) of the repeated experiments. The significance of differences among means of samples was determined using one-way ANOVA with Tukey's post hoc tests.

## Abbreviations

P/O: pollen-ovule ratio; OCl: Outcrossing index; DAP: Days after pollination; MTT: Methyl thiazolyl tetrazolium; SD: Standard deviation.

## Declarations

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### Authors' contributions

QF, ZFX, and CF designed the experiments. CF, YZ, QS, XZ performed the experiments and analyzed the data. CF wrote the manuscript. QF, ZFX, and YBT revised the manuscript. WC, MSC, HH, and BZP participated to analyze the data. All authors have read and approved the final manuscript.

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### Availability of data and materials

All data generated or analyzed during this study are included in this published article.

### Declarations

### Ethics approval and consent to participate

Not applicable. All experimental studies on plants were complied with relevant institutional, national, and international guidelines and legislation.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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## Figures

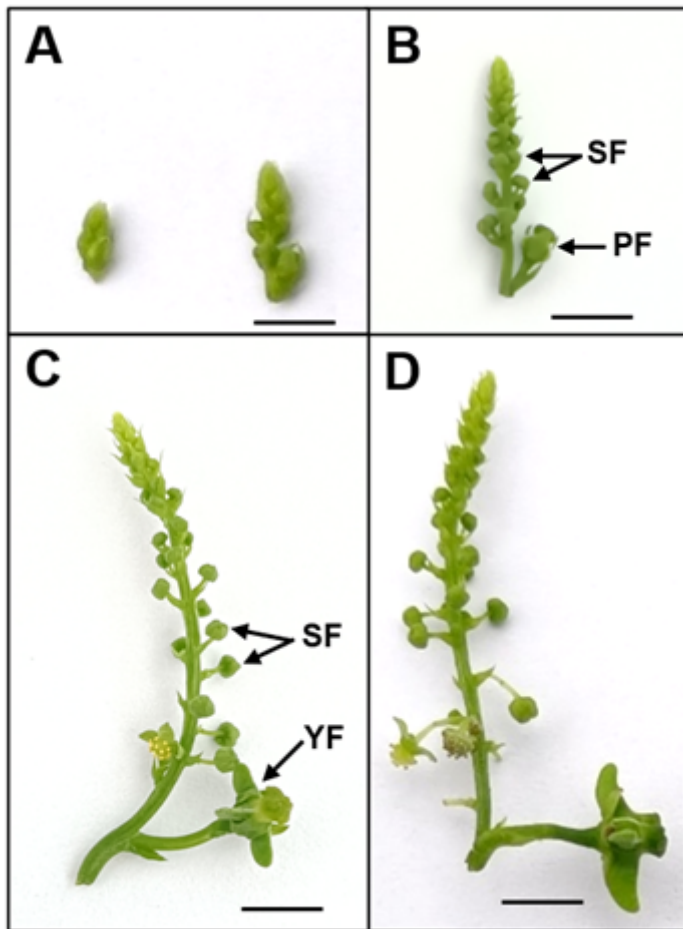


**Figure 1**

Development processes of individual inflorescences of *P. corniculata*. **A.** Budding stage of inflorescence; **B.** Inflorescence with initial blossom stage of pistillate flower; **C.** Inflorescence with initial bloom stage of staminate flower; **D.** Inflorescence with full bloom stage of staminate flowers. SF, staminate flower; PF, pistillate flower; YF, young fruit. Bars=5 mm.

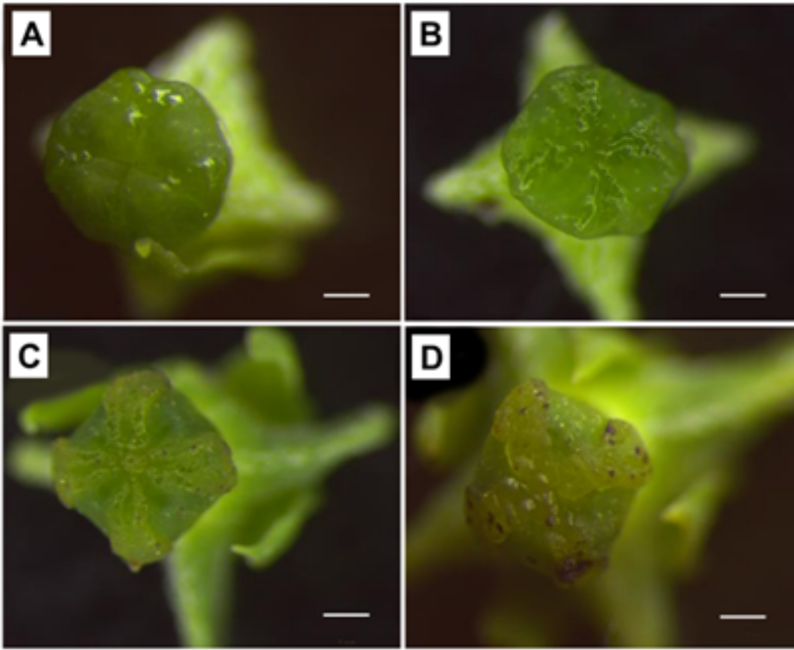


**Figure 2**

Different stages of stigma development in *P. corniculata*. **A.** Indehiscent stigma of young pistillate flower; **B.** Stigma dehiscing on the second day; **C.** Stigma dehiscing on the sixth day; **D.** Stigma dehiscing on the tenth day. Bars=2 mm.

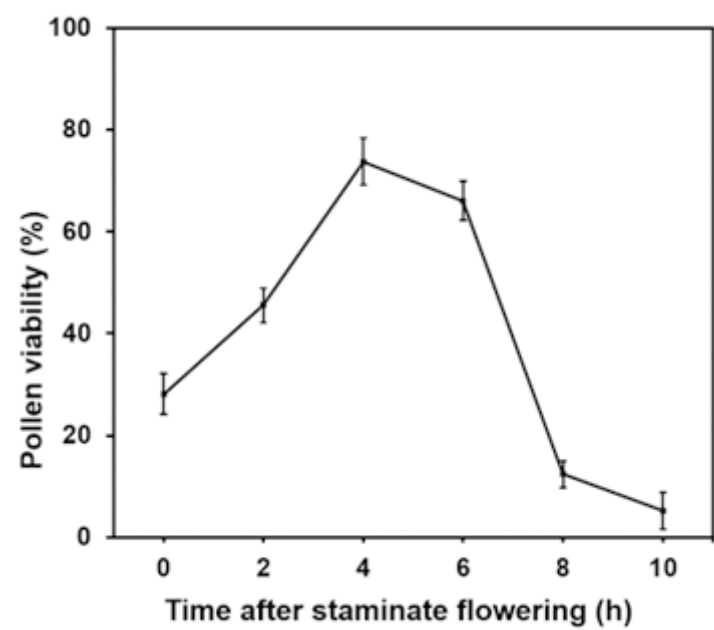


**Figure 3**

Pollen vitality of *P. corniculata* at different times after flowering

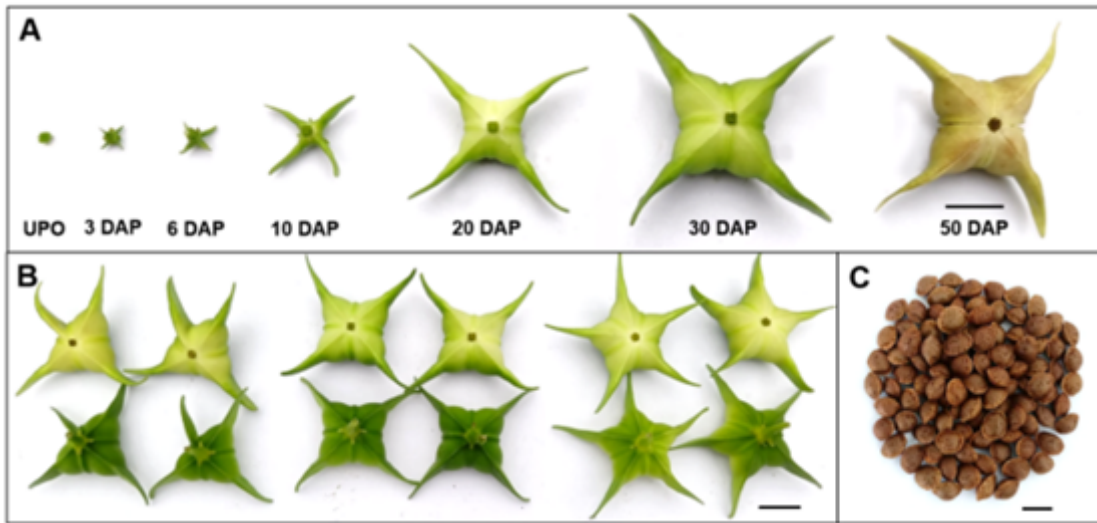


**Figure 4**

Fruit development and seed characteristics of *P. corniculata*. **A.** Different developmental stages of fruits of *P. corniculata*; **B.** Fruit morphology with different number of carpels of *P. corniculata*; **C.** Seed morphology of *P. corniculata*. Bars=1 cm.