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Full Length Research Paper

A study on structural features in early flower development of *Jatropha curcas* L. and the classification of its inflorescences

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***Jatropha curcas* L. produces seed oil that is viewed as having tremendous potential as an economical alternative for diesel fuel. Seed yield, the main factor determining jatropha oil production, is highly associated with flower development, especially with the number of female flowers. However, little was known regarding floral development in this species. Accordingly, studies were undertaken to develop more information on the developmental process of floral organs. The early floral development was divided into 12 phases. The present study illustrated that, the sex differentiation of male or female flowers occurred in phase-VII; earlier phases presented unapparent structural differences. The male flowers always had unisexual tissues during floral development. In contrast, early development of female flowers presented bisexual tissues, with male sexual degradation occurring at the later developmental phases. There was significant location specificity with respect to the inflorescence of male and female flowers. Based on this, the present study combined the total number of female flowers, and divided the inflorescence into three types, which had significant differences in the number of female flowers; they likewise presented different probabilities of occurrence in terms of different growing seasons.**

Key words: Biofuel, flower, inflorescence, *Jatropha curcas*, sex differentiation, scanning electron microscopy.

INTRODUCTION

It has been increasingly apparent that the production of biofuels must originate from non-food crops dedicated solely to energy production. These crops should not compete with food crop production. Genetic improvement of such potential bio-energy-producing crops, such as switchgrass, poplar, and *Jatropha curcas* L. (jatropha) is currently being pursued (Koonin, 2006; Kaushik, 2007; Ranade et al., 2008). Many countries hold high expectations for jatropha as a biofuel source, as it grows well on lands poorly suited for cultivation of food crops.

However, although there is reason to be enthusiastic about jatropha's potential as a biodiesel feedstock, one major obstacle to its current widespread cultivation is its non-domesticated nature. Thus, although jatropha grows abundantly in the wild, its seed and oil yields are considered unpredictable for production. The conditions that best suit its growth remain largely undefined, and the potential environmental impacts of large-scale cultivation are not at all fully understood (Chikara, 2007).

J. curcas L. (Euphorbiaceae) originated in Central America. It is a perennial softwood shrub or small tree that is now fairly well established in most tropical and subtropical areas of the world. The vegetable oil from the oil-rich seeds produced by this plant is known for its medicinal value (Wei et al., 2005), and has long been used around the world as a source of lamp oil and for producing soap (Chikara, 2007). Recently, jatropha oil has been recognized for its suitability for conversion into biodiesel (Solomon, 2002). The plant thrives in a wide range of environmental conditions, from eroded land to reclaimed land. It likewise grows well with limited

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amounts of water, nutrients, and capital inputs. *J. curcas* can be used to rehabilitate wastelands and improve the environment. It can also enhance the quality of rural life by providing new economic resources for marginal farmlands (Openshaw, 2000; Francis et al., 2005).

A number of industrial companies have launched plans and have started to develop new industries based on the jatropha oil (e.g., biofuel production). For instance, China National Offshore Oil Corporation, one of the largest state-owned oil companies in China, has begun to build a six-ton biodiesel factory in the country's Hainan province based on the use of jatropha material. Recently, Air New Zealand has completed a two-hour test flight powered by a 1:1 blend of biodiesel made out of jatropha oil and conventional jet fuel (http://www.bloomberg.com/apps/news?pid=20601081&sid=aMBr28xXRhTE&refer=australia).

From a botanical perspective, the production of seed oil depends on flower production and seed set. However, in jatropha, seed yield, which is highly correlated to the number of female flowers, is currently considered as a limiting factor. The ratio of female to male flowers is low in most of the available germplasm. Therefore, increasing the number of female flowers through breeding and management is essential for the success of seed oil production on a large scale. The aim of the present study is to further investigate the morphological development of the male and female flowers, the number of female flowers per inflorescence, and the types of inflorescences. This information would help in the genetic improvement for oil yield and the mechanism studies of sex differentiation and the objective setting of breeding and cultivating in this plant.

MATERIALS AND METHODS

The experiment was conducted in the provinces of Sichuan, Yunan, and Hainan of China (26°56′N, 101°68′E; 19°25′N, 110°18′E; N22°95′, E104°29′) from 2007 to 2008, with two observation sites located in each province. The trees for this study were 5 to 8 year old. The reproductive body samples (about 300) were collected every two days, from floral tissue emergence to the flower senescence. Flowers were divided into male and female, and were then analyzed by scanning electron microscopy (SEM).

For SEM, fresh flower tissue was fixed overnight in 2% glutaraldehyde in phosphate buffer, pH 7.2 to 7.4 (Wang, 2006), dehydrated in a graded ascending ethanol series, and dried at the critical point with CO_2. Samples were mounted onto SEM stubs with epoxy or carbon-coated tape, and sputter-coated with a 25 nm layer of gold or gold-palladium in a sputter coater. They were then viewed with an S-450 SEM (Hitachi, Japan) running at an accelerating voltage of 5 to 20 kV. To reveal the organs developing underneath, floral structures were dissected.

A total of 5,000 *J. curcas* inflorescences were observed. Observations were initiated in mid-March when inflorescences started to form, and ended in mid-December when new inflorescences had ceased to occur from 2007 to 2008. The number, location, and array of female and male flowers in the inflorescence were recorded for all of the observed inflorescences. Data were statistically analyzed using variance analysis of Statistical Analysis System (Meyes et al., 2009).

RESULTS

Flower structure and location features

To study the structural features of the development process of early flowers of *J. curcas* and the structural differences in their sex differentiation, it is first necessary to clarify the structural features of mature flowers. Typically, mature flowers are unisexual, both male and female flowers having five sepals, five petals, and five glands (Figures 1A, B, C and D). In addition, male flowers have 10 stamens divided into upper and lower layers (Figure 1A), while female flowers have three carpels and two split stigmas (Figure 1C).

The jatropha plant produces flowers on a racemose inflorescence with a dichasial cyme pattern (Solomon and Ezradanam, 2002). A study of the inflorescence of *J. curcas* has shown that the rachis of *J. curcas* develops its branches several times and, in an inflorescence, female flowers would be located between two branches of their inflorescence, while male flowers flourish at ends of the plants' branches (Figure 1E).

Different structural characteristics of flowers in different phases

When the plants of *J. curcas* grow into a flower-matured state, under appropriate environmental conditions, their inflorescence meristem would be differentiated at the top of their branches, which begin their reproductive growth. For a better analysis of the structural characteristics of male and female flowers, the present paper divides the early flower development into 12 phases in accordance with the early or late developmental time of calyx, petal, glands, stamen, and pistil, taking note of the division system established in Arabidopsis (Smyth et al., 1990) and *Silene latifolia* (Grant et al., 1994).

Phase-I to phase-II

In the first two phases, young inflorescences of *J. curcas* cannot be seen from the plant surface, since they are enveloped by young leaves. When the meristem at the top of the branches forms a rounded protrusion, plants begin to shift from vegetative growth to reproductive growth. Such meristem would further elongate to form the inflorescence primordia (Figure 2A), indicating the beginning of phase-I. Then, further differentiation and development of inflorescence primordia would proceed; that is, a number of young inflorescence branches are differentiated on the inflorescence rachis, the completion of which sets off phase-II (Figure 2B). At this phase, each branch is composed of the central flower primordium and the inflorescence meristem on both sides, which is differentiated once again by the number of new inflorescences in the branches. Such developmental

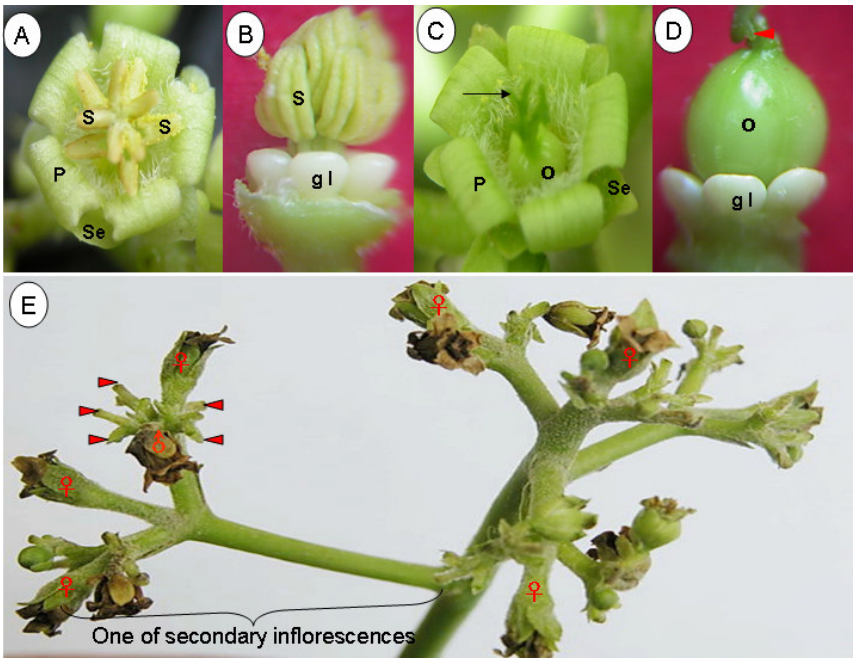

Figure 1. The character of *J. curcas* flowers. (A) and (B) A male flower: ten stamens (s), five petals (p), five sepals (se) and five glands (g). (C) and (D) A female flower: ovaries (o), three styles, two splits each (arrowhead) five petals (p), five sepals (se) and three of five glands (g). (E) Structure of an inflorescence (arrowheads show the pedicels of the fallen male flowers, ♀:female flower, ♂ :male flower)

process forms a number of branches when the inflorescences are matured (Figure 1E).

Phase-III to phase-VI

Along with the further development of flower primordia of phase-II, the sepal primordia begin their formation. When the primordia of five calyxes appear without the occurrence of petal primordia, it is then defined as phase-III (Figure 2C). In phase-III, inflorescence tissues are visible at the top. When the primordia of five petals are formed at the base of the pentagram-like flower tissues, it is referred to as phase-IV (Figure 2D).

Then, at the base of the flower tissues and the inner part of petal primordia, further differentiation and growth out of five gland primordia are seen. This stage is now defined as phase-V (Figure 2E). At this phase, the elongated flower meristem appears like a circular truncated cone. Here, the five petal primordia can be observed as having a slower growth just prior to the formation of the pistil or stamen primordia. At phase-VI, there is a formation of ten nearly oval-shaped stamen primordia divided into upper and lower layers, with each layer having five oval-shaped stamen primordia (Figure 2F), which is consistent with the structure of mature male flowers (Figure 1A). SEM has shown that the early development of female flowers also presents 10 stamen primordia, which is followed by further development of the female structure (Figures 3A and B). Therefore, the formation patterns of female and male organs in phase-III and phase-VI are the same, specifically, from the calyx to the petal and stamen gland formation.

Phase-VII to phase-XII

Immediately after phase-VI, female and male flowers begin to show their developmental differences, mainly, in the form of changes in the flower apical meristem. If such apical meristem undergoes further differentiation and protuberance, then female flowers are formed, and the formation of protuberance of the apical meristem is defined as phase-VII of the female flowers (Figure 3A). In this period, the apical meristem of male flowers no longer exhibits differentiation or elongation, but its stamen primordia continue to develop, with their top surrounded by nearly oval-shaped stamen primordia and the center taking a planer shape (Figure 3D). Carpel tissues are likewise differentiated, developed, and formed from the top protrusion of the meristem. The carpel primordia emergence represents phase-VIII of the female flower development (Figure 3B), while the structural emergence of heart-shaped stamen primordia coming from a nearly oval-shaped form is defined as phase-VIII of male flower development (Figure 3E).

Then, carpel tissues further differentiate and develop to form ovule primordia, now referred to as phase-IX of the

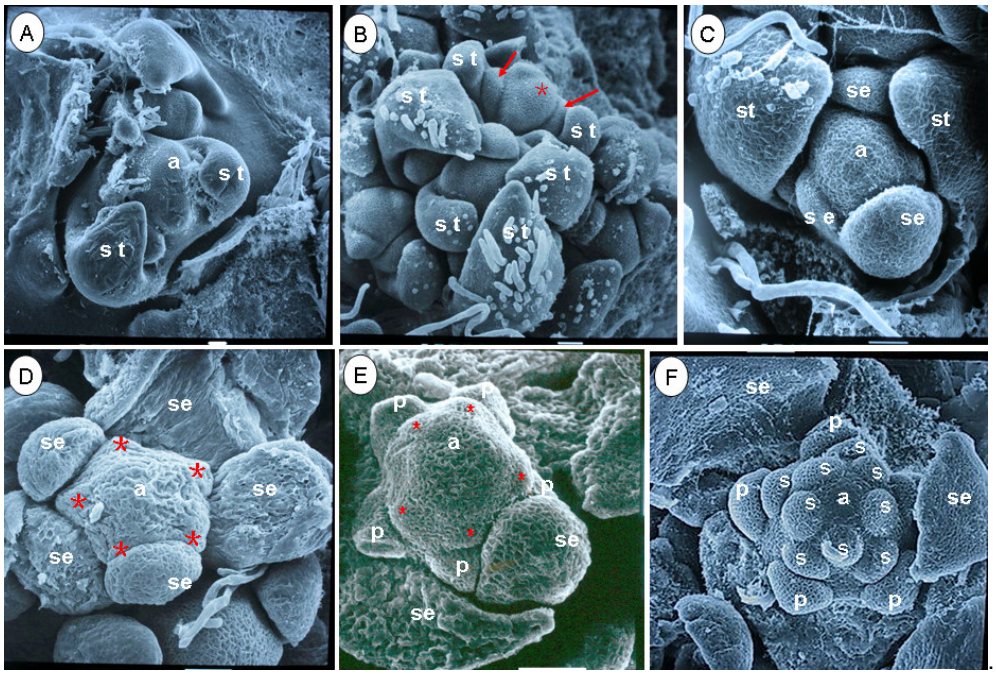

Figure 2. Scan of *J. curcas* L. flowers of phase-I to phase-VI. (A) The main inflorescence primordia emerges, bracketed by a pair of stipules in phase-I. (B) A branch of inflorescence composed of two lateral inflorescences (arrowheads) and a central flower (*) in phase-II. (C) Sepal primordia enclose a near spheroid meristem in phase-III. (D) Reproductive meristem like pentagram as five-petal primordia (*) arise in phase-IV. (E) Five glands primordia (*) arise at the base of circular-truncated-cone-like floral meristem in phase-V. (F) Stamen primordia arise in phase-VI. a: apical meristem, st: stipule primordium, se: sepal primordium, p: petal primordium. Bars = 50 µm.

female flowers (Figure 3C). This period is defined as the formation of ovule primordia, and a female flower usually is shown to have three ovules. In phase-IX, male flowers transform their stamen primordia from heart-shaped to shallow cleft-shaped (Figure 3F). The formation of each carpel's top central protuberance (developing into style in the future) is defined as phase-X of female flowers (Figure 4A). In this period, the stamens of male flowers develop from shallow cleft-shaped into strips, while their pollen sac begins to take shape and each stamen takes on the formation of four pollen sacs (Figure 4D).

At phase-XI, a distinctive developmental feature of both female and male flowers emerges, particularly, their quickly growing glands (Figures 4B and E). The female flowers' stigma is formed and carpel development allows the relative separation of the ovule and the surrounding tissues (Figure 5A). Meanwhile, each anther of the male flowers is divided into two parts, each part having two rooms with pollen grains separated from each other (Figures 4E and 5B). Growing to phase-XII, the female flowers' stigma is observed to grow towards development and maturation (Figure 4C), while the male flowers' filament grows toward elongation and most pollen grains present are round (Figures 4F and 5C). Not long after this period, elongation of flowers, maturity of gametophytes, and blossoming of flowers occur.

Sexual differentiation

According to the above-mentioned division of developmental phases of early flowers of *J. curcas*, no obvious structural difference between male and female flowers can be observed before phase-VII, as similar tissues and organs are formed, such as the sepals, petals, glands, and stamen primordia. In the latter period of phase-VI, the flowers whose top starts to elongate (Figure 3A) will grow as female flowers, while those without further differentiation and development (Figure 3D) will grow into male flowers. Thus, in the early stage of development of female flowers, bisexual tissues are prominent (Figure 3A, B and C). In the subsequent development, male tissues undergo abortion (Figure 4A and B), and traces of the stamens of abortion can still be seen in the nearly mature female flowers (Figure 4C). Since there is no emergence of female tissues in male flowers, their development process has always been unisexual (Figures 1A, 3D, E, F; 4D, E and F). Thus, there are mainly two modes for the formation of unisexual flowers of *J. curcas*: one is the formation of female flowers after the later abortive development of male tissues, and the other is the formation of male flowers subject to the early adolescence when there is no occurrence of female primordia.

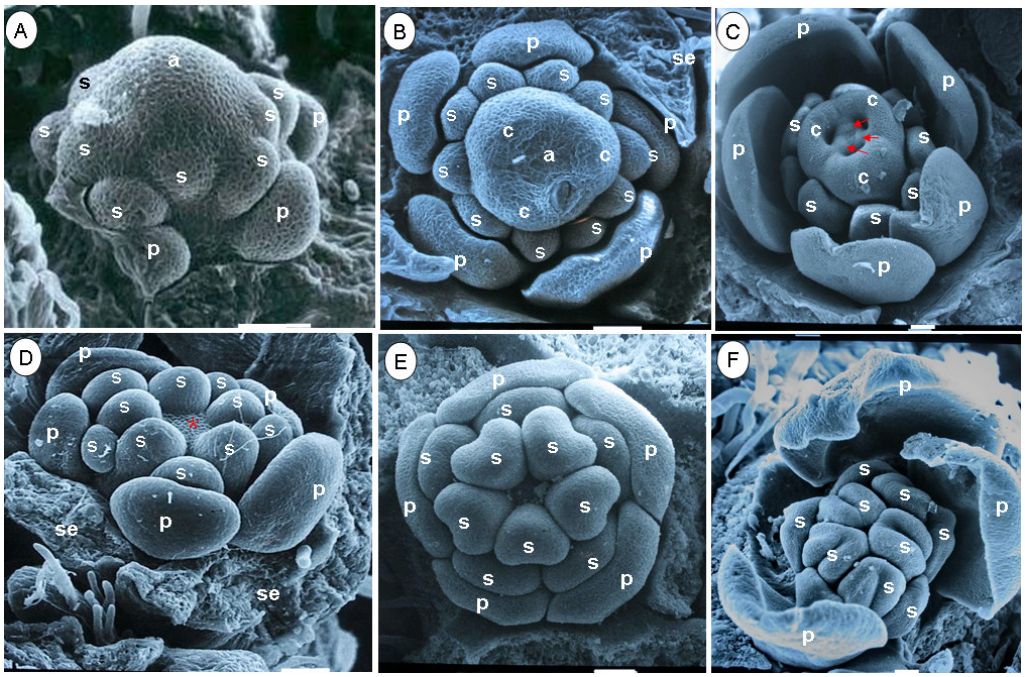

Figure 3. Sex differentiation of *J. curcas* L. flowers in phase-VII to phase-IX. (A) The apical meristem is elongated during phase-VII of a female flower. (B) Three carpels are established with ten stamens on the base in phase-VIII of a female flower. (C) Three ovule primordia (arrowhead) emerge in phase-IX. (D) The apical meristem (asterisk) is arrested and only stamen primordia exist of the two gender organs in phase-VII of a male flower. (E) The heart-shaped stamen primordia are formed in phase-VIII of a male flower. (F) Stamen primordia are transformed from heart-shaped to shallow cleft-shaped structure in phase-IX of a male flower. a: apical meristem, p: petal, s: stamen, c: carpel. Bars = 50 μm

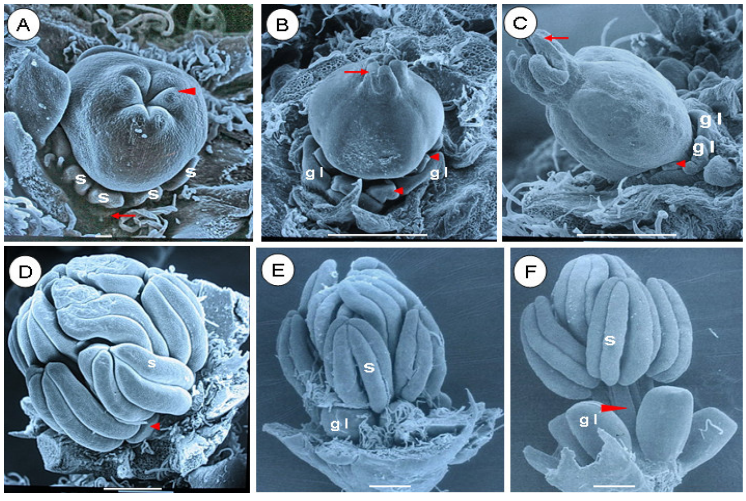

Figure 4. The development of *J. curcas* L. flowers of phase-X to phase-XII. (A) The carpel's top protuberances (bold arrowhead) indicate the formation of style primordial with the undeveloped gland (thin arrowhead). (B) Stigma is formed (thin arrowhead); the glands grow up in a short period; the aborted stamen (bold arrowhead) is visible. (C) The floral stigma (thin arrowhead) develops further to be ready to receive the pollens in phase-XII with the trail of aborted stamen (bold arrowhead). (D) The stamens develop from shallow cleft-shaped into strips with the undeveloped glands (bold arrowhead). (E) The glands grow up quickly in phase-XI of male flowers. (F) The filament (bold arrowhead) grows toward elongation in phase-XII. s: stamen, gl: gland. Bars = 50 μm in A and D, bars = 500 μm in B, C, E and F.

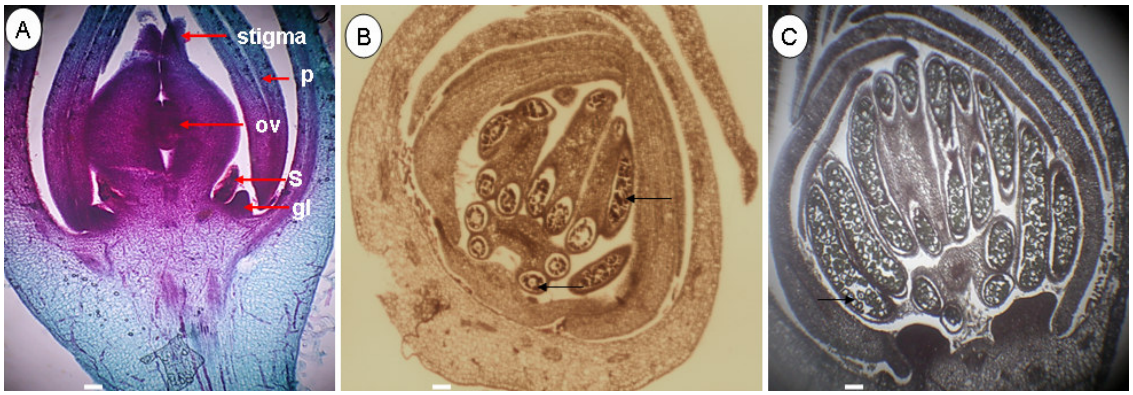

Figure 5. The sections of *J. curcas* L. flowers in phase-X and phase-XI. (A) The stigma is formed and the ovule (the future seed) is relatively separated from surrounding tissues; the aborted stamen is visible at the base of the ovary outside. (B) The pollen grains (arrowhead) start to be formed in phase-X of male flowers. (C) Most pollen grains (arrowhead) develop into round shape in phase-XI of male flowers. p: petal, s: stamen, gl: gland, ov: ovule bars = 500 µm.

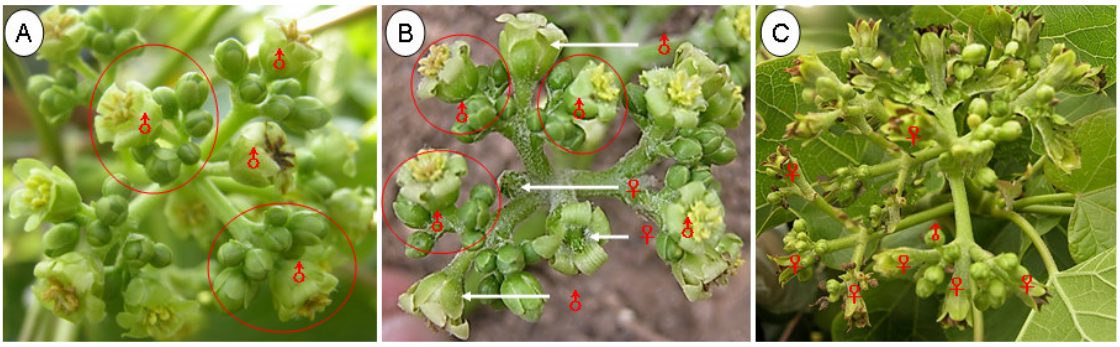

Figure 6. The configuration of *J. curcas* inflorescences. (A) male-type inflorescence: all flowers are male. (B) A middle-type inflorescence. (C) A female-type inflorescence: all the places of female sites are occupied by female flowers. ♀: female flower, ♂: male flower.

Inflorescence classification and ratio

As mentioned above, male and female flowers of *J. curcas* have their significant location features with respect to their distribution on inflorescence. For the early inflorescence development, the main inflorescence rachis is divided into a few branches, with each branch composed of a central flower and two sub-branches of inflorescence meristem generated in their maturation (Figure 2B). Then, sub-branches of inflorescence are differentiated into another central flower and lower sub-branches of inflorescence meristem, which no longer appears to be differentiated in inflorescence branches; its end part presents the formation of three to six flowers (Figures 1E and 6B). In this way, mature inflorescence is developed with obviously branched inflorescence rachis. A large number of observations indicate that, the flowers formed on the third branch of the sub-branched inflorescence rachis would only grow into male flowers (Figures 1E, 6B and C), and the locations of inflorescence growing flowers are defined as male sites. The top of the main inflorescence rachis and the central location where the first and second sub-branches start may form the female flowers (Figures 1E and 2B), and are thus defined as the female sites. Based on the gender of female sites of inflorescence, the present paper divides inflorescence into three types that can, to some extent, reflect the proportion of male and female flowers of inflorescence, especially the number of female flowers, as well as reflect the number of their final fruits. When all the female sites of inflorescence are presented as female flowers, then such inflorescence should be divided into female-type inflorescence (Figure 6C). When all of these sites are presented as male flowers, then all the flowers of such inflorescence are male flowers, referred to as male-type inflorescence (Figure 6A). When the number of female flowers at the female sites is between the female-type inflorescence and the male-type inflorescence (that is, the female sites of female flowers at an inflorescence, do not grow all female flowers or male flowers), then such intermediate type is called middle-type inflorescence (Figure 6B).

Table 1. Variance analysis of the female flower number per inflorescence between the female-type and middle-type inflorescences.

Source	DF	Type III SS	Mean square	F value	Pr > F
Inflorescence type	1	518.57	518.57	8418.31	<.0001**
Site	5	0.27	0.054	0.88	0.56
Year	1	0.024	0.024	0.39	0.56
Inflorescence type × site	5	0.023	0.005	0.08	0.99
Inflorescence type × year	1	0.018	0.018	0.29	0.61
Site × year	5	0.296	0.059	0.96	0.52
Error	5	0.308	0.062		
Corrected total	23	519.51			

×: interact, **: significantly different, DF: Degree of freedom.

Table 2. Variance analysis of the male: female flower ratio per inflorescence between the female-type and middle-type inflorescence.

Source	DF	Type III SS	Mean square	F-value	Pr > F
Inflorescence type	1	1976.9	1976.9	42295.6	<.0001**
Site	5	0.097	0.019	0.41	0.82
Year	1	0.014	0.014	0.3	0.61
Inflorescence type × site	5	0.069	0.014	0.29	0.9
Inflorescence type × year	1	0.025	0.025	0.54	0.49
Site × year	5	0.276	0.055	1.18	0.43
Error	5	0.23	0.047		
Corrected total	23	1977.61			

×: interact, **: significantly different.

For ease of calculation, the branching inflorescence generated from the sub-branches of the main inflorescence rachis is called secondary inflorescence (Figure 1E), which can further branch several times during the development. Each secondary inflorescence has three female sites (Figures 1E and 6C), while the number of the secondary inflorescence's male sites are different on different inflorescences. The statistical analysis of the present study has indicated that, the secondary inflorescence has an average of 20 male sites. Statistics also show that a single inflorescence has an average of six secondary inflorescences. The variance analysis indicated that there are great differences, reaching to an extremely remarkable level ($Pr > F$, <0.0001), between the female-type and the middle-type inflorescences with respect to the number of female flowers and the proportion of female and male flowers of a single inflorescence (Tables 1 and 2). However, for different years and different sites, there is no obvious difference ($Pr > F = 0.56$ and 0.56) between the female-type and the middle-type inflorescences with respect to the number of female flowers and the proportion of female and male flowers per inflorescence (Tables 1 and 2). Meanwhile, the male-type inflorescence's female flower number is zero and the proportion of staminate flowers and female flowers is infinity. Therefore, there is extremely remarkable difference with respect to a single inflorescence's female flower number and the proportion of female and male of the three types of inflorescences. The female flower number and the proportion of female and male flowers for three types of inflorescences are shown in Table 3. Herein, the proportion of male and female flowers is 25:1 on average. The proportion of male and female flowers and the female floral number of the female-type inflorescence are about 15:1 and 16.5, respectively, while these are 20:1 and 7.2 for the middle-type inflorescence.

Through the observation for more than two years, it has been discovered that different types of inflorescences have different proportions (Table 3). To illustrate, female-type inflorescence presents the smallest (0.09%) proportion, while middle-type presents the largest (74.9%). Moreover, different proportions occur in different seasons for the same type of inflorescence. Since jatropha is expected to blossom several times in a year, for ease of research, the average of three times of blossoms per year was assumed for the analysis. Based on this assumption, it is found that female-type and

Table 3. Number of male and female flowers in three types of inflorescences.

Types	Inflorescence number	The ratio in all inflorescences	Female flower number	Male flower number	Male: female ratio	Average female flower number per inflorescence
Female-type inflorescence	450	0.09	7425	65295	8.79	16.5
Middle-type inflorescence	3475	0.749	25020	674100	26.94	7.2
Male-type inflorescence	1075	0.215	0	218225		0

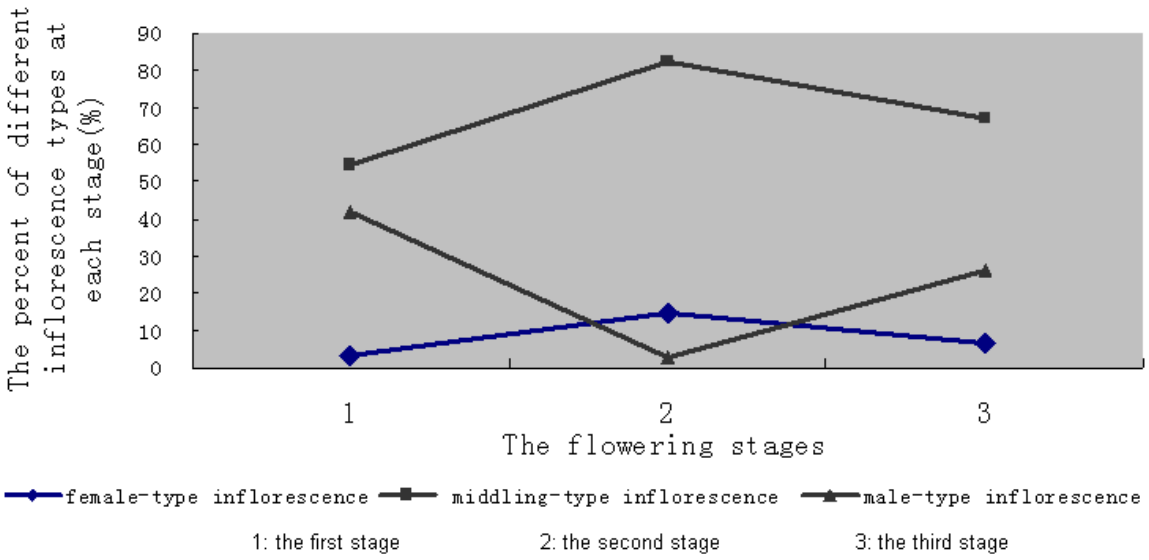

Figure 7. Different inflorescence types at different flowering stages in *J. curcas*.

middle-type inflorescences reach the highest proportion of “one-time blossom” during the middle period, while the proportion for the “first-time blossom” reaches the highest for the male-type inflorescence (Figure 7).

DISCUSSION

The development of flowering plants is an orderly progression from the embryo to the mature plant through continuous organ formation from meristems (Krizek and Fletcher, 2005). Most studies indicate that both sex organ primordia occur in the early developmental stages in unisexual flowers (dioecious or monoecious); the selective developmental arrest of preformed organ primordia is the most common method for generating unisexual flowers (Delong et al., 1993; Grant et al., 1994). However, some studies do not support the idea that the production of unisexual flowers is controlled by selectively activating or inactivating homeotic gene functions (Dellaporta and Calderon-Urrea, 1993). Such as in *Spinacia oleracea*, *Mercurialia annua* and *Pistacia vera*, only one kind of sex primordia are formed during the flower development process (Durand and Durand, 1984; Sherry et al., 1993; Wannan and Quinn, 1991).

Our results show that there are two modes of formation of unisexual flowers in jatropha. In female flowers, bisexual tissues are present and the androecium selectively arrests its future development. In male flowers, only one kind of sex tissue is present, and thus, there is no selective arrest of stamens or pistils. Besides, male and female flowers have similar tissues and organs before female primordia emergence, and these development characteristics were also reported in *Clusia valerioi* (Hochwallner and Weber, 2006). Based on this organization of the flower sexes in jatropha, we propose that there are factors that regulate sex differentiation in this plant, which may selectively affect the action of homeotic genes in one whorl, such as only restraining the initiation of a gynoecium meristem in male jatropha

flowers.

Previous studies have shown that the pollination in jatropha flower is done by insects that are attracted by the nectar produced by the flower (Bhattacharya et al., 2005; Solomon and Ezradanam, 2002). The nectar secretion coincides with the time when pollens have developed in the anthers and the stigma has become receptive, which is important for pollination. However, the structural basis for this to happen has not been clarified. During floral development, glands emerge at phase-V, but they only mature at phase-XI when the flowers are ready to open. Therefore, just before the flower opens, glands mature to release an aroma to attract insects. If glands were to mature and release scent at an earlier stage, the attracted insects would be blocked by the petals enclosing the reproductive organs. By the time flowers do open, the release of scent from the glands would have ceased, and insects are then less attracted to the flowers. As such, it is essential that the glands do not mature and release scent until the flowers open, especially since insects are attracted at a correct time in order to complete the pollination.

The present research has shown that the female and male flowers of *J. curcas* have remarkable position location features; that is, female flowers are located at the female sites, male flowers are located at the male sites and the proportion of an inflorescence's male sites and female sites is approximately 20:1. Therefore, for *J. curcas*, male flowers dominate the female flowers. The study also indicates that female flowers do not appear at the male sites, but male flowers may develop at the female sites. This explains why an inflorescence has less number of female flowers in the actual production. The related investigation has demonstrated that each inflorescence has an average of seven female flowers but an inflorescence has an average of 18 female sites (6 secondary inflorescences on the average). Thus, enhancing the number of female flowers at the female sites of an inflorescence would be an effective method for transforming the male-type inflorescence into the middle-type or the female-type inflorescence to improve the output.

The present research has demonstrated that female sites would not change with time and locations for different types of inflorescences. Thus, all may be divided using this kind of inflorescence type division with respect to different years and different locations. The research also demonstrates that the male-type inflorescences (zero in terms of female flower number) accounts for 30%; female-type inflorescences are relatively fewer at 8%. Since production of female flowers forms the premise for production of seeds, the output of seeds of *J. curcas* is less ideal in actual production. Moreover, different seasons result in different proportions of inflorescence types. Considering the present research, female-type and middle-type inflorescences occur mostly in seasons of appropriate temperature and rainfalls. Such features are presented especially in relation to temperature, since the first occurrence of blossom is within the period of February to March, right after the end of the previous year and at a time of low temperature dormancy. The third occurrence of blossoms (possibly the fourth or fifth time in some places) occurs in the period of October to November when temperature gradually drops. We have also observed that the right amount of rainfall may possibly enhance the number of female flowers for each inflorescence, while too much or too less would not favor the occurrence of female flowers (data not shown).

In conclusion, the present study provides new details regarding floral development and inflorescence types (related with the female number) in *J. curcas* L. The findings reported here would be useful in bringing this species into domestication, thus improving the production of biodiesel and extending its application to other uses.

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