Pollination biology, breeding system and reproductive success of *Adhatoda vasica*, an important medicinal plant

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***Adhatoda vasica* Nees. (Acanthaceae) is an important and widely used medicinal plant. The flowers last for 3–5 days, they are protandrous and pass through three distinct phases: male, bisexual and female. Two species of carpenter bees, *Xylocopa verticalis* and *Xylocopa* sp. are the effective pollinators. Pollen grains are deposited on the dorsal surface of the thorax during *Xylocopa* visit to the flowers in the male phase, and the stigma rubs the pollen-coated thorax and is pollinated when the bees visit the flowers in bisexual and female phases. There is a high level of geitonogamy. Pollination efficiency under field conditions is high (95%). However, fruit set is poor (6%). The species is self-incompatible. None of the self-pollinated flowers sets fruits, but over 50% of the cross-pollinated flowers sets fruits. The results indicate that protandry does not prevent self-pollination, but reduces interference in export and import of pollen. Although the flowers have adapted well to achieve a high level of pollination, reproductive success in terms of fruit set is low, largely due to the limitation of compatible pollen.**

Keywords: *Adhatoda vasica*, breeding system, pollination biology, protandry, *Xylocopa* spp.

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ADHATODA VASICA Nees. (Acanthaceae) is a common perennial shrub that grows on wastelands and gardens in most parts of India, Nepal and parts of Pakistan. It is an important medicinal plant and its roots and leaves are used as anti-asthmatic, anti-spasmodic, bronchodilator and expectorant. Its bronchodialatory effects are due to the presence of alkaloids, vasicine and vasicinone. In the ayurvedic system of medicine, *A. vasica* is commonly known as vasa and has been in use for over 2000 years[1,2]. Many ayurvedic preparations containing leaf and root extracts of *A. vasica* are commercially available. In spite of its importance and wide distribution, no information is available on its reproductive biology, particularly pollination biology. This communication presents the results of studies on reproductive biology of *A. vasica*. The primary objectives were to study floral phenology, identify effective pollinators and to investigate pollination efficiency, breeding system and reproductive success.

Studies were carried out during two seasons (January–March 2007 and 2008) during the peak of flowering on a population growing on fallow land in Odekar Farm near Thovinakere, (13°32'.583N and 77°02'.945E), located about 30 km from Tumkur, Karnataka. Comparative studies were also carried out on another population growing about 4 km away from this population. To study floral phenology, flower buds that would open the next day were tagged ($N = 50 \times 2$) and were kept under observation (every hour on the first day and every morning, noon and evening on subsequent days until senescence) to record the time of anthesis, anther dehiscence and structural changes associated with ageing of flowers. The amount of nectar was measured with 5 μl calibrated microcapillaries (microcaps, Drummonds) and the concentration of sugar in the nectar was estimated with a portable refractometer.

Pollen viability was assessed on the basis of fluorescein diacetate test[3]. Pollen samples were collected in the morning from freshly opened flowers and maintained under laboratory conditions, and were tested for viability each day until they lost viability completely. Stigma receptivity was assessed on the basis of pollen germination following manual pollination. Flower buds that would open the next morning were tagged and bagged. The time and day of their opening were recorded and the samples were kept under observation for their sexual phase. Flowers ($N = 10$ for each phase) were pollinated during male, bisexual and female phases with fresh pollen (collected from another plant) and rebagged. They were excised 12 h after pollination, fixed in FAA and used to study pollen germination using aniline blue fluorescence[4]. The stigmas were observed under a fluorescence microscope and those that permitted pollen germination were considered receptive.

Initial observations revealed that several insects visit the flowers throughout the day from 0600 to 1800 h. The frequency of visits and foraging time were continuously recorded from 0600 to 1800 h for three days (36 h of total

observation). Pollinators were distinguished from floral visitors based on their landing site, method of foraging, their contact with the anthers and stigma, presence of pollen load on their body and their efficiency in transferring pollen to the stigma. For studying the transfer of pollen to the stigma, flowers visited by the insects were excised and observed under the microscope for pollen load on their stigma. During some of their visits, the flowers were observed carefully from a close range to record their reproductive (male, bisexual or female) phase, and the position of the anthers and the stigma during insect foraging. A few pollinators were caught using sweeping net, immobilized in ethyl acetate vapours and used to study pollen load on their body. For studying pollination efficiency under field conditions, the pistils from senescing flowers on the fourth/fifth day of anthesis ($N = 50 \times 3$) in which the corolla had abscised were excised and their stigmas observed under a stereomicroscope for the presence of pollen load.

To study the breeding system, flower buds that would open the next morning were bagged. On the third day of their opening, when the flowers were in the female phase, the bags were opened and the flowers were manually pollinated either with cross-pollen (from another plant) or with self-pollen (from another flower of the same plant) collected from flowers in the male phase and were rebagged. To check the prevalence of apomixis, flowers were bagged after emasculation, and for autogamy flowers were bagged without emasculation. Treated flowers were left on the plant to study fruit set. The fruits were harvested before they shed their seeds, and scored for seed number.

Reproductive success was assessed on the basis of fruit and seed set. As the bracts, bracteoles and sepals are persistent and continue to be green until all the flowers in the inflorescence have opened and the fruits have reached maturity, it was possible to count, from the older inflorescences, the number of flowers produced and the number of fruits developed from each inflorescence. The per cent fruit set under field conditions was calculated by counting the total number of flowers produced in each inflorescence ($N = 30$) and the number of fruits developed. The fruits were excised when they started turning brown and the number of seeds was counted.

A. vasica flowers throughout the year in flushes, although the number of flowers produced in each flush is limited. The peak of flowering is during mid-January to early March, when the whole plant is covered with a large number of flowers. The flowers are typically Acanthaceous and bi-lipped. The two lips of the corolla fuse to form a corolla tube (ca. 1.5 cm long). Violet nectar guides radiating toward the corolla tube are present on the central lobe of the lower lip. Two epipetalous stamens arising from the upper lip gently curve down with greenish anthers. The style in freshly opened flowers is positioned close to the upper lip of the petal. The stigma is terminal, less de-marcated, slightly bi-lipped, and is dry and non-papillate. The ovary is two-loculed, with two ovules in each locule.

Most of the flowers open during the morning hours, although a few continue to open throughout the day. Anthesis is initiated around 0530 h and the flowers are fully open by 0700 h. Anthers dehisce by 0930 h. All flowers remain fresh for three days, irrespective of whether they are pollinated or not. In pollinated flowers the corolla abscises from the thalamus on the fourth day morning and then starts drying up. The ovary and style do not abscise immediately even from those flowers which do not develop into fruits. In most of the flowers, the abscised and dried corolla is seen hanging down along the persistent style for several hours before it falls-off. A small proportion of flowers (ca. 5%) remains unpollinated on the third day and such flowers remain fresh for another two days or until pollinated, before they start senescing. The bract, bracteoles and sepals of all the flowers in the inflorescence are persistent, irrespective of whether they develop into fruits or not. Dried style is also persistent at the tip of the fruits until an advanced stage. Fruits remain green for about 2 weeks and start turning brown. After 4 weeks, the fruits dehisce and release the seeds with limited force. The bracts and bracteoles in the inflorescence fade and dry up after the dispersal of seeds from the dried fruits.

Flowers are distinctly protandrous. Studies on the changes in structural features of the stigma and anthers, pollen viability and stigma receptivity showed that the flower passes through three distinct phases: male, bisexual and female phase (Table 1). Immediately after flower opening, the anthers are the only functional reproductive parts and the stigma at this phase is non-receptive. The style is positioned close to the upper corolla lip with distally pointed non-receptive stigma. The stamens are curved down and the anthers are turgid and positioned below the stigma and style (Figure 1 *a*). Pollen viability is >80% during this phase. Male phase lasts for 24–30 h from anthesis.

The male phase gradually leads to a short bisexual phase. The style, particularly the terminal part, curves down to reorient the distally pointed stigma downwards (Figure 1 *b*). The stigma now becomes receptive. The anthers remain in the same position as in the male phase and continue to be turgid. During the bisexual phase pollen viability is reduced to $27.52 \pm 6.2\%$. Little pollen is left in the anthers during this phase in flowers that were already visited by pollinators in the male phase. However, in flowers that do not receive visitors during the male phase, the anthers offer considerable amount of viable pollen during the bisexual phase. The bisexual phase lasts for 10–12 h.

Later, the flowers become functionally female. In this phase the anthers do not offer any pollen; they shrivel, dry up and gradually the filaments move apart to position the dried anthers away from the bilipped corolla (Figure 1 *c*). In bagged flowers, though the anthers retain most of the pollen grains during the female phase, they are nonviable.

Table 1. Floral features of the three phases of flowers

Feature	Male phase	Bisexual phase	Female phase*
Duration from anthesis (± 4 h)	0–30 h	30–40 h	40–72 h
Period of each phase (± 4 h)	30 h	10 h	32 h
Nature of the style and stigma	Style straight and stigma distally pointed	Terminal part of the style curved and stigma pointed downward	As in bisexual phase
Stigma receptivity	Non-receptive	Receptive	Receptive
Nature of anther	Fresh, green and turgid with abundant viable pollen	Slightly faded but turgid with some viable pollen	Shrivelled, brown without any viable pollen; gradually the filaments also dry up and spread out
Pollen viability	>80%	ca. 30%	Not viable

*In a small proportion of flowers that do not get pollinated by 72 h, the female phase continues until pollination or for another 48 h.
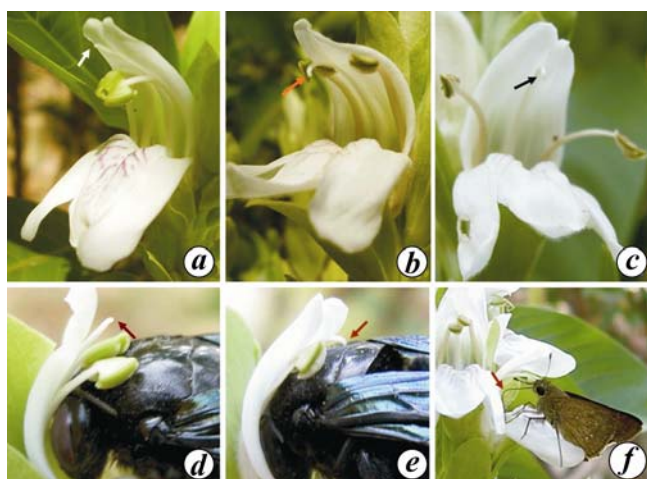

Figure 1. Flowers of different phases in *Adhtoda vasica*. *a*, Male phase. The arrow points to the non-receptive stigma. *b*, Bisexual phase. The stigma (arrow) has curved down. *c*, Female phase. The stigma is in the same position as in the bisexual phase, but the anthers have dried and moved away. *d*, *Xylocopa* sp. visiting the flower during the male phase. Although the anthers rub the thorax of the insect, the stigma (arrow) does not come in contact with the insect's body. *e*, *Xylocopa* sp. visiting the flower during the bisexual phase. Both the anthers and stigma (arrow) come in contact with the body of the insect. *f*, A butterfly (Bevan's swift) robbing nectar by inserting its probosis (arrow) into the corolla tube.

Figure 2. Frequency of visits by *Xylocopa* sp. to flowers of *A. vasica* over time.

The stigma continues to be receptive and is pointed downward. The female phase lasts for 24–32 h in pollinated flowers. However, in non-pollinated flowers the female phase continues for another 48 h.

Flowers of *A. vasica* offer pollen as well as nectar as reward. Maximum amount of nectar (5.9 ± 1.17 µl) accumulates in the corolla tube by 11.30 h. The nectar is viscous and the concentration of sugar in the nectar is 46%. Because of the repeated visits of the pollinators, there is no measurable nectar in the flowers by evening. However, additional nectar is secreted during the night; the amount of nectar present in the flowers on the second and third day was 4.85 ± 1.37 µl and 3.15 ± 0.57 µl respectively. A small amount of nectar is present in unpolli-
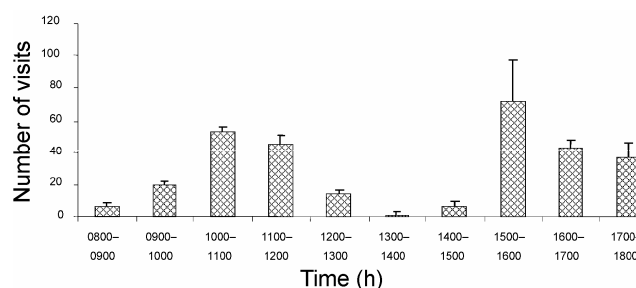
nated flowers even on the fourth and fifth day (3.15 ± 0.57 µl and 3.02 ± 0.61 µl respectively).

A total of five species of insects visit the flowers: two species of carpenter bees (*Xylocopa verticalis* and *Xylocopa* sp.), *Apis cerana*, a butterfly (Bevan's swift, *Pseudoborbo bevani* Moor.) and *Amegilla* sp. Both the species of *Xylocopa* come in contact with the anthers as well as the stigma and contain abundant pollen load on the dorsal surface of the thorax. The visits of both the species start around 0800 h and continue until 1800 h. The frequency of visits of both the species showed one peak in the morning hours and one in the evening hours (Figure 2). They enter deep inside the flower and forage nectar for 2–10 s. Rhythmic movement of the abdomen is clearly seen during foraging. They visit all three phases of the flowers without any preference during each of their foraging bouts. Often the visits of both the species overlap.

The positions of the anthers and stigma were closely observed in several flowers ($N = 30$) during bee visits. In the male and bisexual phase of the flowers, the two anthers invariably rub the upper surface of the thorax of the bee and considerable amount of white pollen gets deposited on the thorax. However the anthers of the third phase are already dry, shrivelled and hardly contain any pollen; even in those which contain some pollen, they are non-viable. The anther filaments in the female phase

gradually move apart, thus positioning the anthers away from the path of the visiting bee. During *Xylocopa* visits, the distally pointed stigma of the flowers in the male phase remains straight and is placed at some distance above the insect; it does not touch the body of the insect (Figure 1 *d*). Thus no pollination can take place during the male phase. However, downwardly curved stigma of the flowers of the bisexual and female phases invariably rubs the pollen-covered thorax of the insect and gets pollinated (Figure 1 *e*). Both the pollinators visit one flower after another of the same plant, covering flowers of all the phases before they fly to the next plant. During each foraging trip, the number of flowers visited by *Xylocopa* sp. on each plant was 33.25 ± 9.40.

A. cerana, *Amegilla* and the butterfly are not the pollinators as they never come in contact with the stigma. *A. cerana* does not visit fresh flowers. It always visits flowers in which the corolla has abscised from the thalamus and is tilted to one side, thus exposing the base of the corolla tube and the lower part of the ovary. *A. cerana* invariably forages left-over nectar present inside the lower part of the abscised corolla tube. The butterfly lands on the lower lip of the flower and inserts its long proboscis into the corolla tube (Figure 1 *f*), and forages nectar for 5–15 s. *Amegilla* lands on the anther and forages pollen; it spends a short time (<2 s) on each flower. Thus *Amegilla* is the pollen robber and the butterfly the nectar robber; they do not bring about pollination.

Flowers of the male, bisexual and female phases visited by the bees were marked ($N = 25$ for each phase); they were excised and their stigmas checked for pollen load. None of the flowers visited in the male phase was pollinated, whereas 93.3% of the visited flowers of the bisexual and female phases were pollinated. Open pollination efficiency was assessed by excising persistent style and stigma from senescing flowers ($N = 110$) and scoring their stigmas for pollen load under a stereomicroscope; 95% of the flowers turned out to be pollinated. Average number of pollen grains deposited on the stigma of each flower was 14.19 ± 7.9.

Details of fruit set are presented in Table 2. Fruit set under open-pollination was poor. Only about 6% of the flowers sets fruits, with an average of 1.1 seeds per fruit. None of the emasculated and bagged flowers as well as those bagged without emasculation sets fruits (Table 2), indicating the absence of apomixis or autogamy in this species. Also, none of the manually self-pollinated flowers sets fruits, while over 50% of the cross-pollinated flowers set fruits. The results clearly show that the species is strictly self-incompatible.

The flowers of *A. vasica* are functional for three days, irrespective of the time of pollination. During this period they pass through three sexual phases: male, bisexual and female. The flowers are distinctly protandrous. The male phase lasts for 24–30 h. During this period over 80% pollen grains is viable and the stigma is nonreceptive, positioned away from the path of the insect entry and exit. However, the male and the female phases overlap (bisexual) for 10–12 h during which the stigma is receptive and the flowers offer pollen, although the degree of viability is less than 30%. Such an overlap has also been reported in *Burchardia umbellata*[5]. The bisexual phase leads to the female phase which lasts for 24–36 h. In flowers which do not get pollinated by 72 h, the female phase continues for another 48 h.

Temporal (dichogamy) separation of pollen availability and stigma receptivity is quite common in species with bisexual flowers[6–8]. In dichogamous species, protandry is more common when compared to protogyny[8]. Dichogamy has been traditionally interpreted as a devise to prevent inbreeding[8,9]. This interpretation has been questioned in recent years based on the occurrence of self-incompatibility in the same proportion in both dichogamous and non-dichogamous species[10]. Recent studies indicate that dichogamy, particularly protandry reduces interference in pollen export and receipt[6,8,10–12]. In *A. vasica* too, although protandry is distinct and prevents autogamy to a large extent, it is ineffective in preventing geitonogamy. Because of the availability of a large number of fresh flowers on each plant, the pollinators tend to move from one flower to the other of the same plant, resulting in extensive geitonogamy. However, protandry does prevent interference with pollen export and receipt, since most of the pollen is removed from anthers during the male phase when the stigma is positioned away from the path of the bee visit. Protandry and position of the stigma during the male phase also prevent the clogging of non-receptive stigma with self-pollen. This seems to be important in *A. vasica*

Table 2. Fruit and seed set in treated flowers

Treatment	No. of flowers pollinated	No. of fruits developed (%)	Average no. of seeds/fruit
Bagged without emasculation	30	0	–
Bagged after emasculation	30	0	–
Self-pollination*	30	0	–
Cross-pollination*	30	17 (56.6)	1
Open pollination	119	8 (6.72)	1.1

*Pollinated during the female phase of the flower.

since the stigma is small and clogging of the stigma with self-pollen may prevent the functioning of the cross-pollen, which may land on the stigma at a later stage.

The flowers seem to have adapted well to achieve pollination success. All flowers remain functional for three days, irrespective of their pollination status. If pollination is not achieved in three days, unpollinated flowers remain functional until pollination or up to two more days and continue to attract pollinators. Both the *Xylocopa* species visit each flower repeatedly on all the days until the flowers remain fresh and act as efficient pollinators. Most of the pollen export is completed on the first day itself, when the flower is in the male phase. Flowers of the subsequent days receive the pollen as their stigmas are receptive, and get pollinated.

Controlled pollinations clearly showed that *A. vasica* is self-incompatible, as none of the self-pollinated flowers sets fruits. Manual cross-pollination results in over 50% fruit set. This is another example of co-occurrence of protandry and self-incompatibility which has been reported in a large number of species[6]. In *A. vasica*, fruit set under field conditions was only about 6%. Pollen limitation (quality/quantity) is one of the main causes for low fruit set[13–17]. Self-incompatible species tend to be more pollen-limited than self-compatible species[5]. Our reslults clearly show that in *A. vasica* limitation of pollen quality and not the quantity is a major cause for low fruit set. Flowers of *A. vasica* attract a large number of visits of the two pollinator species and almost all the flowers are visited many times during the female phase of the flower. Pollination efficiency under field conditions is high (95%) and the average number of pollen grains deposited on the stigma of each flower was 14.19 ± 7.9. As the number of seeds developed in each fruit is generally one (out of four ovules), the number of pollen grains deposited on the stigma is adequate to induce fruit set in all pollinated flowers. In *Trichanthera gigantea*, another member of Acanthaceae, deposition of at least eight pollen grains on the stigma was sufficient to induce fruit set[18]. Each plant of *A. vasica* produces over 100 fresh flowers with adequate nectar reward at any given day during the peak of flowering. Pollinators tend to move from flower to flower of the same plant, thus bringing about geitonogamous pollination. As the species is strictly self-incompatible, geitonogamous pollinations do not result in fruit set. There is limited interplant movement of the pollen and the number of flowers that get cross-pollinated with compatible pollen would be limited. Thus although the species is well adapted to achieve effective pollination, its reproductive success in terms of fruit set is poor due to limitation of compatible pollen.

1. Chopra, R. N., *Indigenous Drugs of India*, Academic Publishers, Calcutta, 1982.
2. Singh, A. P., 2008; C:\Papers in press\Adhatoda\Adhatoda Internetr\Adhatoda vasica-Therapeutic Monograph.htm
3. Heslop-Harrison, J. and Heslop-Harrison, Y., Evaluation of pollen viability by enzymatically induced fluorescence: intracellular hydrolysis of fluorescein diacetate. *Stain Technol.*, 1970, **45**, 115–120.
4. Shivanna, K. R. and Rangaswamy, N. S., *Pollen Biology: A Laboratory Manual*, Springer-Verlag, Berlin, 1992.
5. Ramsey, M. and Vaughton, G., Pollen quality limits seed set in *Burchardia umbellata* (Colchicaceae). *Am. J. Bot.*, 2000, **87**, 845–852.
6. Bertin, R. I., Incidence of monoecy and dichogamy in relation to self-fertilization in angiosperms. *Am. J. Bot.*, 1993, **80**, 557–560.
7. Bertin, R. I. and Newman, C. M., Dichogamy in angiosperms. *Bot. Rev.*, 1993, **59**, 112–152.
8. Routley, M. B., Bertin, R. I. and Husband, B. C., Correlated evolution of dichogamy and self-incompatibility: A phylogenetic approach. *Int. J. Plant Sci.*, 2004, **165**, 983–993.
9. Lloyd, D. G. and Schoen, D. J., Self- and cross-fertilization in plants. I. Functional dimensions. *Int. J. Plant Sci.*, 1992, **153**, 358–369.
10. Lloyd, D. G. and Webb, C. J., The avoidance of interference between the presentation of pollen and stigmas in angiosperms. I. Dichogamy. *N.Z. J. Bot.*, 1986, **24**, 135–162.
11. Harder, L. D., Barrett, S. C. H. and Cole, W. W., The mating consequences of sexual segregation within inflorescences of flowering plants. *Proc. R. Soc. London, Ser. B*, 2000, **267**, 315–320.
12. Barrett, S. C. H., Sexual interference of the floral kind. *Heredity*, 2002, **88**, 154–159.
13. Burd, M., Bateman's principle and plant reproduction: the role of pollen limitation in fruit and seed set. *Bot. Rev.*, 1994, **60**, 83–111.
14. Dudash, M. R. and Fenster, C. B., Multiyear study of pollen limitation and cost of reproduction in the iteroparous *Silene virginica*. *Ecology*, 1997, **78**, 484–493.
15. Knight, T. M. *et al.*, Pollen limitation of plant reproduction: Pattern and process. *Annu. Rev. Ecol. Evol. Syst.*, 2005, **36**, 467–497.
16. Wilcock, C. and Neiland, R., Pollination failure in plants: why it happens and when it matters. *Trends Plant Sci.*, 2002, **7**, 270–277.
17. Hirayama, K, Ishida, K. and Tomaru, N., Effects of pollen shortage and self-pollination on seed production of an endangered tree, *Magnolia stellata*. *Ann. Bot.*, 2005, **95**, 1009–1015.
18. McDade, L. A., Pollination intensity and seed set in *Trichanthera gigantea* (Acanthaceae). *Biotropica*, 1983, **15**, 122–124.