

Reproductive biology of *Withania ashwagandha* sp. novo (Solanaceae)

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Abstract

Withania ashwagandha Kaul (Solanaceae) is an annual plant species of immense medicinal importance. It is a repository of a large number of pharmacologically active secondary metabolites known as withanolides. Evidence for the delimitation of the species from *W. somnifera* has been provided by our group using multidisciplinary approaches. Knowledge of reproductive biology of medicinal plants is crucial for improvement, effective conservation and management plans to evolve genetically superior varieties. The present paper reports our findings on the floral biology, pollination behavior and breeding system of *W. ashwagandha* in natural populations grown at our experimental field under near natural conditions. Flowering (peak) takes place during April–July and anthesis occurs between 08:00 and 11:00 h. The period of stigma receptivity coincides with anther dehiscence. Fruit set on pollination treatments ranged from 90.8% (passive autogamy), 72% (assisted autogamy), 30.30% (xenogamy), and 56.50% (geitonogamy) through 50.40% (open pollination). Xenogamy brings about very low fruit set, seed-set and seed germination percentages. It is inferred that *W. ashwagandha* is predominantly an autogamous and self-compatible species. Self-compatibility is mainly accomplished due to close proximity of stigma and anthers. This work is the first report on the reproductive biology of *W. ashwagandha* and will be useful for conservation and development of improved varieties of this multipurpose herb.

Highlights

- ▶ Reproductive biology of *W. ashwagandha* Kaul was reported first time.
- ▶ We report the species to be highly self compatible due to close proximity of stigma and anthers.
- ▶ The period of stigma receptivity coincides with anther dehiscence.
- ▶ Fruit set on pollination treatments ranged between 90.8% (passive autogamy) and 30.30% (xenogamy).
- ▶ Xenogamy brings about very low fruit set, seed-set and seed germination percentages.

Keywords: *Withania ashwagandha*; Floral biology; Pollination biology; Withanolides; Autogamy

1. Introduction

Understanding pollination and breeding systems that regulate the genetic structure of populations is important for determining the reproductive constraints in conservation and management (Kaye, 1999 and Koul and Bhatnagar, 2007). Besides quality improvement of medicinal plants, cultivation of medicinal plants is a challenging task because less is known about their reproductive and seed biology (Anderson and Hill, 2002, Anderson et al., 2006, Neal and Anderson, 2005 and Kaul et al., 2005). Equally important is the pollination behavior of the species which is an integral part of reproductive biology.

The genus *Withania* is represented by 26 species worldwide. India is a host to only two species, *W. somnifera* and *W. coagulans*. A variety of breeding systems ranging from obligate selfing to obligate out-crossing operate in different species of medicinal plants including *Withania* (Lewis and John, 1963, Lalonde and Roitberg, 1994, Kaul et al., 2005, Lattoo et al., 2007 and Singh, 2009). Each one of these systems is accompanied by a syndrome of floral and other characters which favor either autogamy, geitonogamy, xenogamy or a blend of these. *W. ashwagandha*, has a similar floral architecture as well as mating system to *W. somnifera*. Reproductive biology and breeding systems of *W. somnifera* and *W. aristata* has been extensively studied (Kaul et al., 2005, Lattoo et al., 2007, Anderson et al., 2006, Singh, 2009 and Mir et al., 2012).

Our research support the delineation of *W. ashwagandha* from *W. somnifera* based on morphology, chemical profiling, crossability features, AFLP fingerprinting and ITS analysis (Mir et al., 2010 and Kumar et al., 2011). Earlier, Kaul (1957) and Atal and Schwarting (1962) suggested that *W. somnifera* is an amalgamation of two or more taxa. Kaul (1957) proposed a separate species status, *W. ashwagandha* to the cultivated forms in the *W. somnifera* germplasm. Subsequently Sastry et al. (1960) and Dhalla et al. (1961) conducted anatomical, pharmacognostic and chemical studies on *W. ashwagandha* and confirmed its distinct nature from its ally *W. somnifera*. Negi et al. (2006) also confirmed the distinctness of cultivated types on the basis of AFLP and SAMPL studies.

The species is found only in some parts of Madhya Pradesh, Rajasthan and Andhra Pradesh where it exists in small cultivated patches. *W. ashwagandha* shows very low genetic diversity (Mir et al., 2010 and Mir et al., 2011) which is further shrinking at an alarming rate because of ruinous harvesting practices for the growing demand of the pharmaceutical industry. It forms an essential constituent of over 100 traditional medicinal formulations (Kaileh et al., 2007).

In recent years, there has been a great upsurge in the pharmacological studies of this plant as it has been shown to possess a wide array of therapeutic properties including antitumor, immunoregulatory and anti-inflammatory (Davis and Kuttan, 2002 and Kuttan, 1996). Most of these pharmacological activities are attributed to a novel group of naturally occurring triterpenoid C-28 steroidal lactones called withanolides. One of the well known withanolides, Withaferin A emerged as an important therapeutic molecule of *W. ashwagandha* due its anticancer properties (Koduru et al., 2010, Lee et al., 2010, Mayola et al., 2011 and Yang et al., 2011).

Keeping in view the tremendous medicinal importance and a highly restricted distribution of this species, present investigations were envisaged to study the pollination behavior and reproductive biology of the species for progressiveness of conservation, management and genetic improvement.

2. Materials and methods

The present study was conducted at the experimental field station of Indian Institute of Integrative Medicine (IIIM) Jammu (32°44'N and 74°55'E and 400 m above the sea level with a temperature range summer (30–44 °C), winter (5–20 °C) and total annual rainfall of 506 mm). Field studies were carried out during 2008–2011. The institute has a very rich germplasm collection of 'ashwagandha'. Inflorescences at the pre-anthesis stage were kept under observation. Emasculation of buds at pre-anthesis stage was done without damaging pistil. The emasculated buds were bagged with perforated wax coated paper bags which were secured at their open ends with thread to prevent contamination. Open flowers close to the emasculated buds were removed. However some flowers were bagged without emasculation to serve as pollen donors for geitonogamy and xenogamy experiments. Depending on pollination treatments flowers could be classified into six categories: (i) Apomixis – anthers removed and flowers bagged to prevent pollination, (ii) Passive Autogamy – intact flowers bagged to ensure self-pollination, (iii) Assisted Autogamy – intact flower buds bagged and pollen grains manually transferred from their anthers to stigma on the next day when it becomes receptive, (iv) Geitonogamy – pollen grains of other flowers of the same plants dusted on the stigma, (v) Xenogamy – pollen grains of other plants of the same population dusted on the stigma and (vi) Open pollination – plants tagged but not bagged. Each of these treatments was based on varied number of crosses and repeated thrice. For manual pollination, pollen grains of dehisced anthers of donor flowers were collected and transferred to receptive stigma of the receptor flowers which were again covered with perforated wax coated bags for the next three days. Receptivity of stigma was tested by H₂O₂ method (Dafni, 1992).

Manual pollinations were conducted between 09:00 h and 11:00 h during peak flowering period for four consecutive years, 2008–2011. Twenty plants with triplicate (60 plants each year) were selected randomly at maturity for morphological details (size and color of flowers, petal, stamens, pollen grains, ovary, style, stigma, ovules and number of flowers/flowering shoot, etc.). Floral architecture and phenological data were correlated with breeding system. These included flowering time, flowering duration, anthesis, stigmatic receptivity, anther dehiscence, relative length and position of stigma and anthers during floral ontogeny, fertilization, seed set and fruit maturation. To measure size of pollen grains, ovules, etc. a calibrated ocular micrometer was used. To estimate total pollen per flower, mature anthers were collected 1–2 h prior to anther dehiscence, put in Ependorf tubes containing 1 ml of a mixture of lactic acid:glycerin (3:1) and kept in the refrigerator (4 °C) till use. A sample of known volume was placed in a hemocytometer where pollen grains were counted; this value was used to estimate the total number of grains per flower. Percentage pollen viability was estimated by 1% TTC (2,3,5-triphenyl tetrazolium chloride) test (Stanley and Linskens, 1974). Deeply stained pollen grains were treated as viable whereas shrunken and poorly stained as nonviable. Percentage pollen germination was tested from the time of anther dehiscence to 24 h. Pollen grains showing emergence of pollen tubes were recorded as germinated. Scanning Electron Microscopy was

undertaken to study the detailed structure of pollen surface. For this pollen grains were acetolyzed and mounted on a glass plate according to the method of Caccavari and Dome (2000). Thereafter, plates were prepared for electro-micrographs under Scanning Electron Microscope (JEM-JEOL-100 CX II EM with ASID) at 40 kV. Ovule number was obtained from dissections of ovaries under a stereoscopic microscope. Pollen–ovule (p/o) ratio was determined by dividing the number of pollen grains by the number of ovules per flower as described by Cruden (1977) and the mean value of three replications was taken as the correct ratio. Flower visitors were recorded during peak flowering time. Fruit set became visible 3–4 days after fertilization. However percentage fruit set was computed after 40 days. Seeds obtained from the various pollination treatments were germinated in seed germinator at 25 °C and 24 h dark period suitable for this species (Mir et al., 2012). However, the apomixis treatment did not result any fruit or seed set. Difference in percentage fruit set and seed germination were analyzed with paired ‘*t*’ test using passive autogamy as control (Mahajan, 1989).

3. Results

W. ashwagandha is an annual, evergreen, tomentose medicinal herb that grows unto 40–50 cm. It has ovate, sub acute, stellately pubescent leaves with undulate margin and inconspicuous veins. Six to seven shoots arise from the crown. In the present sample flowering started in the first week of March and lasted till September with a peak period between April and July. Usually 5–8 flowers were present in axillary clusters out of which only one or two flowers were found to be open. Flowers were small (0.5–0.7 cm), sub-sessile, complete, pentamerous, actinomorphic, hermaphrodite and hypogynous. Sepals 5 (0.4–0.6 cm), persistent, gamosepalous, dull green, boat shaped with valvate aestivation enlarging with the development of fruit and finally covering it. Petals 5 (0.4–0.5 cm), lurid green colored, gamopetalous, campanulate and arranged in valvate aestivation. Mean floral life span was 8 days ($n = 50$ flowers).

Each flower bore five epipetalous and polyandrous stamens and an exerted pistil (Fig. 1). Anthers were oblong, dithecous, basifixed and introse and dehisced longitudinally. On the inner side, they were in touch with stigma that ensured sufficient pollen deposition. Pollen grains were yellow, smooth-walled, sticky and measured $28 \pm 0.07 \mu\text{m}$ (Table 1). There were 4650 ± 201.5 pollen/anther and $23,250 \pm 275$ pollen/flower. Pollen viability was $82.4 \pm 1.13\%$ (Table 1). The pollen was trizonocolporate and the exine pattern was scabrate–granulate (Fig. 1). The average number of ovules/flower was 38 ± 1.20 and the p/o ratio worked to 611:1. Pistil had a wet, capitate, greenish and papillose stigma. The relative length and close proximity of anthers to the stigma coupled with synchrony between stigma receptivity and anther dehiscence indicates the species to be self-pollinated and autogamous. The ovary is bilocular with a large numbers of ovules arranged in axile placentation.

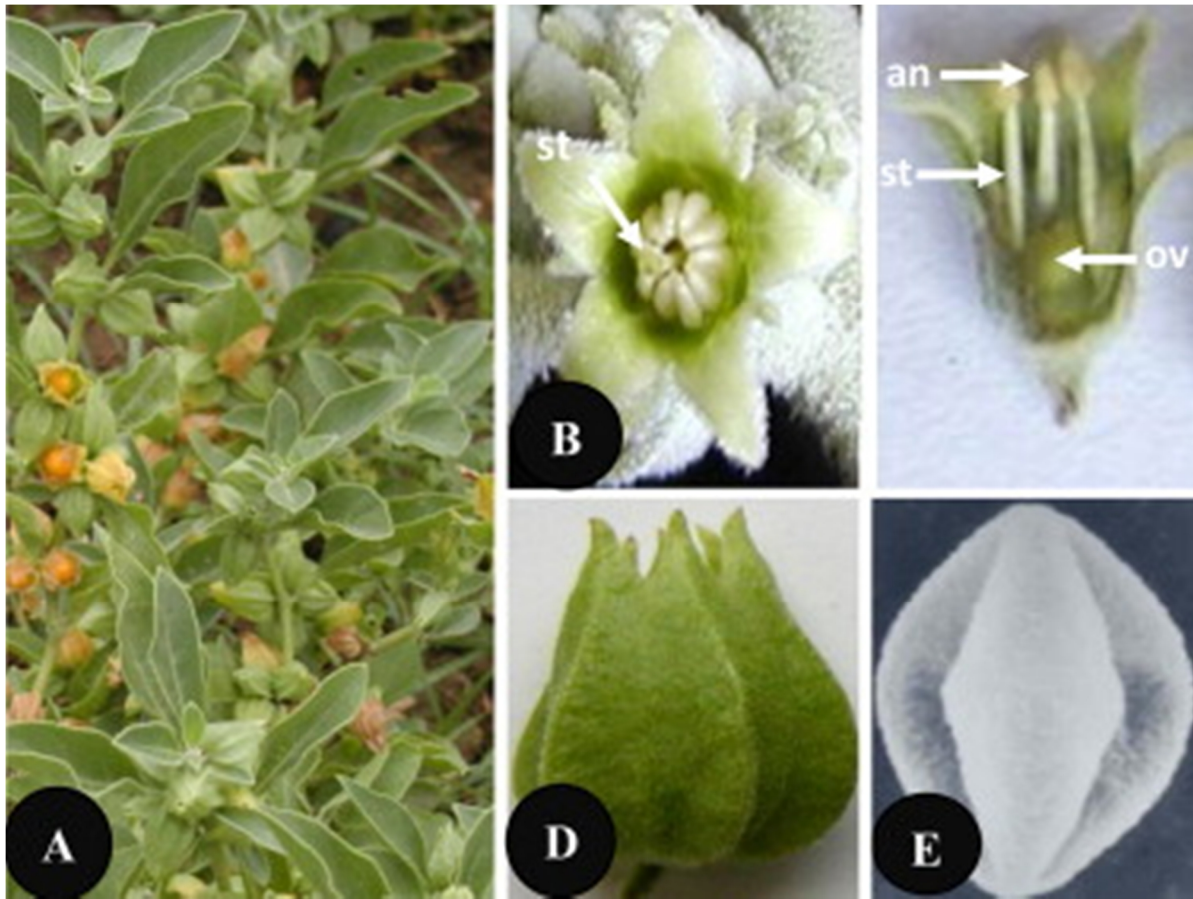


Fig. 1. *Withania ashwagandha* showing the presence of flowers and young fruits. (A) Fruiting twig with orange berries, (B) Single flower showing arrangement of pistil and stamens and greenish receptive stigma (stg), (C) LS of flower 2 days after anthesis shows dehiscent anthers (an), elongated stamens (st) and ovary (ov), (D) Elongated fruiting calyx (Initially fruit is covered with green colored calyx which change into brown on maturation, and (E) Scanning electron micrograph of pollen.

A close perusal of floral phenology revealed that the expansion of petal lobes marked the beginning of anthesis which occurred between 09:00 h and 11:00 h. *Apis dorsata*, *Apis florea*, and butterflies were some common visitors despite the scentless nature of the flowers. Insect visits commenced early in the morning with a peak activity from 8:00 to 12:00 h. During the pre-anthesis phase, the carpel was longer than stamens but by the time anthesis began, stamens had increased in length and exceeded the length of the carpel. The filaments were inserted into stamens or stirrups which were fused with the corolla tube. Stamens (filament base) was addressed to the ovary and a groove between each stamens allowed nectar to flow upwards from the nectary located at the base of the ovary. After anthesis, the petals were retained for about three days after which they turned brown and withered. Fruiting commences simultaneously with flowering and berries took 30–40 days to ripen. However, maximum fruiting occurred during April–July. The fruiting calyx is elongated and prominently ribbed. The berries were invariably yellow and the seeds were dry in texture (33–36 seeds/berry). Details of reproductive features of the species are presented in Table 1.

Table 1. Reproductive characters of *W. ashwagandha*.

S. No.	Parameter	Observations
1.	Flowering season (peak)	April–July
2.	Flower type	Chasmogamous, pentamerous
3.	Flower color	Lurid yellow
4.	Flower odor	Negligible
5.	No. of flowers/node	3–6
6.	Anthesis	8:00–11:00 h
7.	Anther dehiscence time	8:00–11:00 h (two days after anthesis)
8.	Anther dehiscence pattern	Longitudinal (inside)
9.	Peak stigma receptivity	synchronous with anther dehiscence
10.	Stigma type	Pappilose wet type
11.	No. of anther/flower	Five
12.	No. of pollen grains/anther	4650 ± 201.5 (20)
13.	No. of pollen grains/flower	23,250
14.	Pollen viability (TTC test)	82.4 ± 1.13 (2320)
15.	Pollen shape	Spherical-slightly triangular
16.	Pollen size (µm)	28.0 ± 0.07
17.	No. of ovules/flower	38 ± 1.20 (25)
18.	p/o ratio	611:1
19.	Berry	Orange
20.	Fruiting calyx	Elongated
21.	Seed	Non-oily
22.	No. of seeds/berry	34 ± 3
23.	Flower length before anthesis (µm)	5.03 ± 0.022
24.	Flower breadth before anthesis (µm)	4.50 ± 0.022
25.	Flower length after anthesis (µm)	5.33 ± 0.018
26.	Flower breadth after anthesis (µm)	5.23 ± 0.020

Data averaged from 20 replications. Values in parenthesis are number of observations.

Manual pollination of flowers with self pollen resulted in 90.8% fruit set which was only 65% in active autogamy and 56.5% in geitonogamy and 50.4% in open pollination experiments, whereas only 30.3% of fruit set was observed in xenogamy experiments (Table 2). The percentage of seed set per berry among the treatments varied significantly ($p \leq 0.05$) with the minimum number ($23 \pm 2.3\%$) shown by xenogamy treatments. Seeds formed via pollination experiments were evaluated for germination. Autogamously formed seeds showed significantly higher germination percentage of 70.3% in passive autogamy and 68% in active autogamy than geitonogamy and open pollination (60% each), however seeds obtained from xenogamy treatment showed the minimum germination percentage of 40.3% (Table 2).

Table 2. Details of pollination treatments, percentage fruit set and their seed germination in *W. ashwagandha*.

S. No.	Treatment applied	Fruit set (%)	No. of seeds per berry	Seed germination (%)
1.	Passive autogamy	90.8 ± 1.68 ^a (n = 200)	35 ± 1.2 ^a	70.3 ± 4.41 ^a
2.	Assisted autogamy	65.0 ± 2.15 ^b (n = 200)	35 ± 2.3 ^a	68.0 ± 4.45 ^a
3.	Geitonogamy	56.5 ± 3.27 ^c (n = 250)	30 ± 1.9 ^b	60.0 ± 2.23 ^b
4.	Xenogamy	30.3 ± 2.71 ^d (n = 200)	23 ± 2.3 ^c	40.3 ± 5.15 ^c
5.	Open pollination	50.4 ± 1.50 ^c (n = 300)	28 ± 1.3 ^b	60.0 ± 2.68 ^b

The differences in percentage fruit set, seed set and germination percentage among the treatments are significant ($p < 0.05$) by paired 't' test. The "n" represents the number of flowers used for the treatment.

4. Discussion

Under experimental condition of the institute, optimum flowering of the species under study was observed during April–July. Flowers were small (4–6 μm), complete, lurid yellow, sub-sessile and bisexual and 3–6 flowers are arranged in axillary cluster or forming umbellate cymes. The pollen grains were trizonocolporate with scabrate–granulate exine sculpturing (Fig. 1) and of uniform size (28.0 μm). The average number of ovules/flower was 38 ± 1.20 . Pollen viability remained approximately the same up to 12 h of storage in 2% sucrose and gradually decreased thereafter. An interesting feature of this species is that anthers dehisced two days after anthesis exactly at the same time when the stigma is receptive (Table 1). Initially the stamens are short but as the anthesis proceeds, they elongate and anthers touch the stigma on the inner side ensuring sufficient pollen deposition on it. Thus, there seems to be a signal mechanism operating at the time of stigma receptivity. An important result of this synchrony and proximity of stigmatic surface with dehiscing anthers is to create ideal conditions for selfing. Similar mechanism of anther-stigma proximity ensuring selfing was also reported in *Petrocoptis viscose* (Guitian and Navarro, 2002). Self pollination predisposing the similar mechanism has also been reported in few other species e.g. *Bixa orellana* (Renata et al., 2006), *W. somnifera* (Kaul et al., 2005, Mir, 2010 and Mir et al., 2012). Similarly high pollen load on the stigma and stiff pollen competition within a flower with about 611 pollen grains available for siring one ovule, greatly reduce the possibility of cross-pollination. Floral features like small size of flowers, dull green color, high fruit: flower and seed: ovule ratios, are also associated with autogamy. High p/o ratio favors for strong out crossing whereas low p/o ratio is in conformity with the prediction for autogamous species (Cruden, 1977). Thus based on the p/o ratio categories as proposed by Cruden (1977) *W. ashwagandha* would be an obligate autogamous species with low p/o ratio of 611:1. The low p/o ratio was also observed by Amorim et al. (2011) in *Passiflora capsularis* and *P. rubra* two obligatory autogamous species. The infrequent visits of insects on flowers and the insignificant pollen load carried by them further reduce the chances of cross-pollination.

Results of pollination experiments showed that whereas 90.8% fruit could develop through passive autogamy, only 65% fruit developed from assisted autogamy. In both these treatments stigma receive pollen from own flowers but in different manners. The manual transfer of pollen

in active autogamy significantly decreased fruit set indicating that pollen grains do not depend on external agency to reach the stigma surface of the same flower. However, the success of assisted autogamy depends upon frequency of pollen transfer and stigma receptivity (Shivanna and Ram, 1993 and Chauhan et al., 2008). Higher fruit and seed set in controlled self pollination over other pollination treatments (Table 2) further confirms that the species is independent of pollinator visits for reproductive success. Nevertheless, the possibility of a degree of cross pollination cannot be ruled out due to prior exposition of stigma. The higher values of fruit and seed set in passive autogamy further indicate that self pollen grow, germinate and penetrate the pollen tubes into the ovules while in other treatments pollen tubes may not penetrate the ovules indicating flower and fruit abortions. Besides timing of pollen maturation and deposition play an important role with only very recently matured pollen working well. Unattractive flowers pre-dispose the species to an insignificant contribution of insect visitors in pollination. This is a common feature of other self pollinating plant species as well (Primack, 1985, Kaul et al., 2005 and Singh, 2009). Studies conducted by Shivanna and Ram (1993) and Chauhan et al. (2008) reported the requirement of pollinator in self-compatible flowers of *Lantana camara* and *Nardostachys jatamansi* respectively. Seeds developed via pollination experiments showed interesting results; seed germination was significantly higher in seeds obtained in autogamy than geitonogamy, open pollination and xenogamy treatments (Table 2).

The self compatible nature of the species was confirmed by a high percentage of fruit and seed set in controlled self pollination (Table 2). Low fruit set on cross pollination after emasculation was another pointer to the partial fixation of autogamy in the species. Flowering plants possess a wide array of morphological and physiological mechanisms that influence mating patterns, particularly the degree of self fertilization (Eckert and Barrett, 1994 and Gituru et al., 2002).

5. Conclusion

A high percentage of fruit and seed set in controlled self pollination experiments in *W. ashwagandha* confirmed its self-compatible nature. Results of pollination experiments also confirm absence of apomixis. The floral architecture especially relative size and close proximity of stigma and anthers predispose the species for self pollination. The species shows rare occurrence in natural habitats; it needs conservation via '*in situ*' as well as '*ex situ*' methods. Large scale cultivation in its natural habitat on one hand and limited exploitation for the market demand on the other is the only option for conservation. Breeding experiments of this medicinally important plant species (especially xenogamy) will open new dimensions for genetic improvement of crop in future. Further, the information generated by the present study will help in development of hyper-productive varieties with respect to withanolides content and root biomass by inter varietal crosses besides being useful for studying the chemogenetic inheritance of withanolides.

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