Temporal sexual maturation and incremental staminal movement encourages mixed mating in *Withania somnifera* – An insurance for reproductive success

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***Withania somnifera* is a multipurpose plant of immense therapeutic value and wide geographic distribution. It exhibits extensive phenotypic and chemical variability. Here we describe a sexual mechanism deployed by the species to make the maximum out of sexual polymorphism. Our results suggest that the species practices mixed mating as a consequence of partial temporal dichogamy of protogynous type under which the receptive stigma remains exserted beyond the undehisced staminal cone to receive cross pollen through insect vectors. In a probable situation of non-receipt of pollen through insect pollinators, autonomous fertilization is guaranteed by the upward staminal increase to form a cone connivent about the receptive stigma. Floral phenoevents suggest that crossing and autonomous selfing are mutually exclusive as the self-pollen arrives late during floral ontogeny. Seed-set efficiency and fruiting success are not influenced by pollen genotypes (self/cross) under different pollination treatments (autogamy, geitonogamy and xenogamy). Preemergent reproductive success averages 37% with open pollination. DNA content (chromatin length 47.51 µm) is distributed asymmetrically among 24 chromosomes ($2n$ = 48). Meiotic system reveals high recombination index (71.2). In accordance with the theoretical predictions for mixed mating, intermediate levels of heterozygosity are maintained. This is illustrated by the proportion of polymorphic loci calculated as Shannon index employing RAPD. High pollen–ovule ratio (631.77), pronounced protogyny, copious nectar production and insect visitation provide for strong outcrossing. However, small flower size, high seed-to-ovule ratio (0.76) and moderate fruit-to-flower ratio (0.49) are in conformity with the predicts of 'reproductive assurance hypothesis'. Mixed mating and the efficient meiotic recombination system provide a reliable strategy that guarantees reproductive success and genetic polymorphism 'with' you or 'without' you pollinator environment.**

Keywords: Mixed mating, reproductive success, sexual maturation, staminal movement, *Withania somnifera* (L.).

WITHANIA somnifera (L.) Dunal (Solanaceae) is a medicinal plant of immense therapeutic value and wide geographic distribution. It is used in a variety of Ayurvedic and Unani medicines[1]. Its therapeutic value is ascribed to various bioactive molecules synthesized and accumulated in its roots and leaves. Some of the major bioactive constituents include steroidal lactones (withanolides and withaferins), alkaloids and saponins. It is often compared with *Panax ginseng* for its rejuvenating properties[2] and is also described as a panacea for various ailments[3,4]. It presents a huge variability in chemical constituents. More than 35 constituents have been isolated and characterized from its roots and leaves[5]. A fascinating array of chemotypes with severalfold variability in bioactive constituents have been reported from different parts of the world[6]. In recent years there has been a spectacular surge in its pharmacological studies. It has been shown to possess adoptogenic, anti-inflammatory, immunosuppressive, immunomodulatory, anticancer, anticonvulsant, hemopoetic and antioxidative properties. It has also been shown to exert positive influence on endocrine, cardiopulmonary and central nervous system[7–9]. One of the bioactive molecules, withaferin-A has been implicated in the inhibition of Cox-2 (cyclooxigenase-2) and also in the immunosuppression of B lymphocyte proliferation[10,11].

It displays an appreciable spectrum of variability in its morphometeric traits. Atal and Schwarting[12] documented five different morphological forms of *W. somnifera* from different populations growing in varied regions of India. The pattern of genetic variation in plant species is determined by its sexual system, which affects the genetic structure and dynamics of populations within the species[13–15]. Mating systems contribute and control the mode of transmission of genes from one generation to the next.

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Sexual systems operating in plant species are varied and accompanied by differences in floral and reproductive features and syndrome of other characters that favour autogamy, geitonogamy, xenogamy or a blend of these. Diversity in sexual systems is also viewed in relation to regulation of genetic recombination. The rate and nature of recombination are determined by breeding and meiotic systems, which are integral components of the sexual cycle and together constitute the 'genetic system' of a species[16–18].

In the backdrop of immense therapeutic value of *W. somnifera*, and the lack of empirical studies and scarce data with respect to its reproductive biology, it was tempting to elucidate its genetic system with an objective to optimize genetic amelioration and effectively conserve its genetic resources.

The present study was thus aimed at the following: (i) to describe the flowering phenology in relation to mating process and breeding system prevalent in the species, (ii) karyotype and meiotic analysis in order to evaluate the contribution of this component of the genetic system to the generation of variation, and (iii) RAPD profile of selfed and open-pollinated progenies to evaluate how the two compare in genetic diversity, i.e. proportion of exclusive polymorphic loci per progeny and the marker utility function by characterizing polymorphic information content (PIC).

Materials and methods

W. somnifera is a perennial, evergreen, tomentose undershrub that can grow up to 1.5 m tall. Plants have multiple shoots diverging from the basal shoot crown and numerous five-merous flowers in umbellate cyme (5–20 flowers/cyme) in the axils of leaves. Flowers are perfect, actinomorphic with erect exserted stigma. Fruits when ripe are red or yellow-coloured berries with numerous seeds. Flowers are small, lurid-yellow, odourless and visited by both oligolectic bees and anthophagous beetles. Inconspicuous flowers are clustered in conspicuous cymes to attract pollinators. Plants and inflorescences are also visited by ants and ladybird beetles; the beetle has a destructive activity. Visiting insects are rewarded with both pollen and nectar. Floral nectary is present at the ovary base. Pollination syndrome is complex in *W. somnifera*. It presents an appreciable spectrum of interseasonal fluctuations in the availability of pollinators. However, it is not comprehensively dealt with in this article. The present study was conducted at Indian Institute of Integrative Medicine (CSIR), Jammu, India (32°44′N, 75°55′E; 305 m altitude), where the annual temperature fluctuates between 5 and 45°C and mean annual rainfall measures up to 113 cm. The material for the present study comprised bulk open-pollinated seeds from 11 accessions. Plants were raised in well-drained sandy loam soil with periodic organic manuring and irrigation under uniform cultivation conditions. The plants were sown in 2.5 × 2.0 m plots at a spacing 50 × 60 cm apart. Phenological observations and data on various reproductive parameters were recorded from live plants over a period of two years. Floral architecture and different phenoevents observed were related to the breeding system. These included anthesis, stigmatic receptivity, anther dehiscence, relative length and position of stigma and anthers during floral ontogeny, fertilization, seed set and fruit maturation.

Stigmatic receptivity was checked using the method of Shivana and Rangaswamy[19]. Stigmas with germinating pollen grains attached to their surface were considered receptive. Stigmatic area was estimated by measuring the appropriate dimensions using ocular and stage micrometer. Lipids in stigmatic exudates were determined[20] by staining stigmas at receptivity with a mixture of Sudan III and IV. Pollen output per flower was calculated by estimating the number of pollen grains per undehisced anther and multiplying by five (number of anthers). Ovule count per ovary was determined by clearing the fresh pistils with 1 N NaOH and staining with 1% aceto-carmine, followed by gentle squashing. The ovule number was counted under compound microscope. Pollen–ovule ratio was obtained by dividing the number of pollen grains by the number of ovules per flower[21]. Pollen dimensions were estimated from freshly prepared mounts using an ocular micrometer. Pollen fertility at shedding stage was estimated using the fluorescein diacetate test[22].

Six pollination treatments were tested to determine the breeding system. These included, (i) intact open-pollinated flowers as control, (ii) emasculated bagged flowers without pollination to check possible occurrence of apomixis, (iii) bagged unemasculated flowers to estimate spontaneous autogamy, (iv) emasculated open-pollinated flowers to determine natural outcrosssing, (v) geitonogamy in bagged emasculated flowers pollinated with pollen from the same plant, and (vi) for xenogamy, emasculated flowers were cross-pollinated with pollen from other plants. Fruit set and seed-set efficiency were determined for all pollination treatments and control. Seed-set efficiency was calculated as the number of filled seeds, divided by seed potential multiplied by 100. Fruit set was calculated as a percentage of number of flowers that set fruit. The index of self-incompatibility (ISI) was determined by dividing the percentage of fruit set through self-pollination to cross-pollination[23].

For hand-pollination treatments of xenogamy and geitonogamy, pollination was carried out by detaching freshly dehisced anthers with fine forceps and tossing the held anther over receptive stigma to ensure sufficient pollen deposition. In emasculated treatments, undehisced anthers were removed from nearly open flowers (anthesis; Table 1) well before stigmatic receptivity. Butter-paper bags were used to cover the inflorescences according to the treatment requirement. For different pollination treatments not

Table 1. Ontogenetic details of flowering from early bud to fruit maturation in *W. somnifera*

Stage	Day*	SD	Description	
			External	Internal
S_1-Early bud	0	–	Lurid-yellow, sub-sessile; corolla invisible, calyx enveloping, mealy tomentose, teeth acute; size 3.6 mm ± 0.13	Staminal cone and pistil at same height, filaments short; microsporogensis-tetrahedral tetrads; nectar absent
S_2-Late bud	3	0.25	Calyx teeth slightly reflexed; petal lobes not expanded; size 4.6 mm ± 0.07	Staminal cone slightly shorter than stigma; pollen grains fully differentiated; nectar secretion begins
S_3-Anthesis**	5	0.33	Anthesis begins; corolla pubescent outside, glabrous inside; size 6.02 mm ± 0.01	Erect exserted wet papillate stigma; staminal cone visible, nectar accumulation
S_4-Stigma receptivity***	6	0.23	Flowers fully expanded; petal lobes recurved; stigma shiny, exserted; anthers undehisced	Papillate stigma covered with copious lipiodal secretions; copious nectar secretion
S_5-Anther dehiscence	8	0.32	Anthers forming a cone connivent about the stigma	Anther dehiscence through longitudinal slits; pollen smooth-walled, tricolpate two-celled; stigma receptive, nectar secretion present
S_6-Fertilization	10	0.34	Dehisced anthers nearly empty, reflexed away from stigma, slightly brown; corolla faded–wilting	Ovary slightly swollen, stigmatic papillae start fading, remnants of exudates present; nectar secretion ceases
S_7-Early fruit maturation	22	2.80	Berry green–enveloped in persistent inflated calyx, calyx conical, rounded, 10-ribbed, pointed with connivent teeth	Berry globose, glabrous with numerous seeds, creamy-white, immature, discoid
S_8-Late fruit maturation	51	4.60	Red or yellow berry enveloped in conical membranous/papery calyx	Berry 4–6 mm in diameter, pulp fleshy, seeds mature, smooth, yellow.

*Start of the given stage and end of the preceding stage (mean days).
**Floral development stage marked for emasculation.
***Stage for hand-pollination.

more than four flowers per plant were marked, except for spontaneous autogamy where whole cymes were bagged. Flowers aborted and/or damaged due to rain, wind, predation and mechanical injury were not included in the sample.

Reproductive success is the product of the fruit-to-flower ratio (Fr/Fl) and seed-to-ovule (S/O) ratio[24]. Fifteen randomly selected individual plants were tagged to measure the reproductive success. To check the natural fecundity, fruit-to-flower ratio was recorded at regular intervals during the annual growth cycle. However, temporal and spatial variation in fruit and seed set was not taken into consideration.

For meiosis, ten plants at random were selected and their young inflorescences at an ideal stage were fixed in ethanol : chloroform : acetic acid (6 : 3 : 1) for 24 h, transferred to 70% alcohol and stored under refrigeration at 4°C. Squash preparations were made following La Cour's 2% aceto-carmine method for meiotic studies.

For mitotic studies tips from roots of field-grown seedlings were excised, washed with water and pretreated with saturated solution of *p*-dichlorobenzene for 3 h. Pretreated material was thoroughly washed with tap water, fixed in ethanol : chloroform : acetic acid (6 : 3 : 1) for 24 h and stored in 70% ethanol at 4°C. For karyotype analysis, root tips were hydrolysed in 1 N HCl and stained and squashed with 2% aceto-orcein. Observations were made from metaphase stage of cells and chromosome measurements were recorded from freshly prepared mounts using ocular and stage micrometer. Battaglia's scheme[25] was employed for classifying the somatic chromosomes. Cumulative sum of the haploid chromosome complement at metaphase was taken as total chromatin length. At least 52 preparations from seven plants in metaphase were used for observations.

To corroborate the genetic consequences of spontaneous self-pollination and natural open-pollination, the self- and open-pollinated progenies were evaluated for polymorphism employing RAPD analysis. The two progenies obtained were sown separately in earthen pots containing a mixture of soil, sand and vermiculate in the proportion of 4 : 2 : 1 under outdoor conditions. Genomic DNA was extracted from leaves approximately 60 days after germination. Sample size was 15 for each group of progenies, and in total comprised 30 extractions. DNA was isolated following the modified procedure of Doyle and Doyle[26]. The yield of DNA was 50–80 $\mu g\ g^{-1}$ tissue and the UV absorbance ratio at 260/280 nm was at least 1.9–2.1. An aliquot of 3 µl of the preparation was checked on 0.5%

agarose gel for purity. In a prescreen with 39 primers based on the amplification of *W. somnifera* plants, nine arbitrary decamer primers (Operon Technologies, USA) were selected for polymerase chain reaction (PCR). These primers produced distinct amplification profiles that were easily scorable. DNA amplification was performed in Master Cycler Gradient (Ependorf, Germany) and the PCR conditions were as follows: 3 min at 95°C, 40 cycles of: 1 min at 94°C, 1 min at 35°C, 2 min at 72°C and as a last step 10 min at 72°C. Twenty microlitres reaction mixture contained 1 × PCR buffer (10 mM Tris-HCl, pH 9.0; 50 mM KCl; 2.5 mM $MgCl_2$; 200 µM dNTP (Promega); 200 µM random primers; 20–30 ng of DNA template and 0.5 U of Taq DNA polymerase (Bangalore Genei, Bangalore). On completion of the programme for amplification of DNA samples, they were stored at –20°C. Separation of DNAs was performed by electrophoresis in 1.5% (w/v) agarose gel. The gel was documented using EDAS (Electrophoresis Documented and Analysis System). All the PCR reactions were repeated at least twice to check the reproducibility.

Amplification profiles were recorded and the size of each fragment was estimated using SEQUAID II (tm) version 2.2, 1987. Amplicons were scored as discrete variables, using 1 for presence and 0 for absence. Amplification profiles were matched to calculate the comparative polymorphism exhibited by the open-pollinated and self-progenies. Genetic diversity was estimated by Shannon index[27]:

$$H = -\sum_{i=1}^{k} pi \ln pi,$$

where H denotes the diversity of RAPD markers in a population, k is the number of bands produced with the respective primer and pi is the frequency of the ith fragment.

PIC was calculated as $PIC = 1 - \Sigma P_i^2$, where P_i is the band frequency of the ith allele[28] and was considered to be $1 - p^2 - q^2$, where p is the band frequency and q is no band frequency[29].

Results

Floral architecture and flowering phenology

W. somnifera produces flowers indeterminately round the year, with peak flowering between March and July. There is strong asynchrony of flowering as the flower opening is staggered, i.e. flowers open sequentially with 1–3 flowers opening per day per cyme. It takes about 7–11 days for all flowers to open in a cyme cluster. Floral architecture of *W. somnifera* is similar to other solanaceous members: a calyx with five fused sepals divided more or less halfway down, tomentose outside and glabrous inside; a gamopetalous corolla with petals divided halfway down with recurved acute tip, pubescent outside and glossy inside. Reproductive apparatus comprises five stamens and a single pistil. Stamens are epipetalous and adpressed around the style forming anther cone. Each stamen bears a long filament terminating in a bilobed anther. Pistil bears proximal glabrous ovary and an erect style terminated by a globose, shiny, wet, papillate stigma.

Inflorescences differentiated as lurid-yellow, sub-sessile buds in bractless umbellate cymes in the axils of opposite leaves on floral branches. Flowers developed over an average period of five days from early bud to anthesis and 41 days were required for fruits to reach maturity. The mean number of days from early bud stage to different flowering stages, including the number of days between stages is summarized in Table 1 and illustrated in Figure 1.

Anthesis initiates with relaxation of petal lobes exposing the exserted stigma. Corolla mouth expands and petal lobes get reflexed and recurved backward to expose the staminal cone (Figure 1 *a*). This gives the flower a campanulate appearance. At anthesis, flower measures 6.02 × 3.5 mm in size and registers no increase in size over the different stages of development. An exserted pistil averages 4.51 mm ± 0.11 and 4.76 mm ± 0.15 at anthesis and anther dehiscence respectively. Adanation of anthers to filament is basal and filaments of all anthers are equal in length. Filaments register nearly one and half-fold increase in length from 1.95 ± 0.03 to 3.84 mm ± 0.11 from anthesis to anther dehiscence. Stigma attains receptivity 48 h (SD = 0.32) prior to anther dehiscence and receptivity lasts for 6–10 h after anther dehiscence. Relative dimensions of stamens and pistil at three developmental stages of flowering are given in Table 2.

Copious lipoidal secretions and germinating pollen grains adhering to the stigmatic surface mark peak stigma receptivity (Figure 2 *c* and *d*). There is temporal separation of about 48 h between stigma receptivity and anther dehiscence; staminal cone attains height of the stigma after two days of stigmatic receptivity. The filaments elongate and anthers form a cone connivent about the stigma (Figure 1 *c*). Anther dehiscence is prompted in this configuration within 1–2 h. Anthers dehisce through longitudinal stromial slits and release their contents on either of the stigmatic side (Figure 1 *d*). Stigmatic area is large enough (0.23 sq. mm ± 0.003) to accommodate about 923 ± 28.43 pollen grains (N = 22) within half an hour of anther dehiscence. Nectar secretion commenced at stage S_2 (late bud stage; Table 1). Copious amount of nectar secretion was coincident with stigma receptivity and accumulated in the campanulate cup around the filament bases. Secretion continued until 10–16 h after anther dehiscence and ceased following fertilization. Anatomically floral nectary is represented by central vesculature surrounded by five radiating parenchyma segments (Figure 2 *b*) and subtended by epidermal layer and a thin cuticle. Nectar seems to move within the grooves between parenchyma rays and exude through stomata.

Following fertilization, ovary registered increase in size and became apparent after 24 h when nearly empty anthers get reflexed away from the stigma (Figure 1 *e*). Corolla shrivelled and abscissed within 2–3 days after fertilization (Figure 1 *f*). Each flower lasted 5–6 days from anthesis to wilting of corolla. Stigma lost its gloss as the stigmatic exudates dried up and papillae collapsed and turned brown (Figure 1 *f* and *g*). Fruits and/or seeds are dislodged with the aid of heat, wind, rain, and predation by insects.

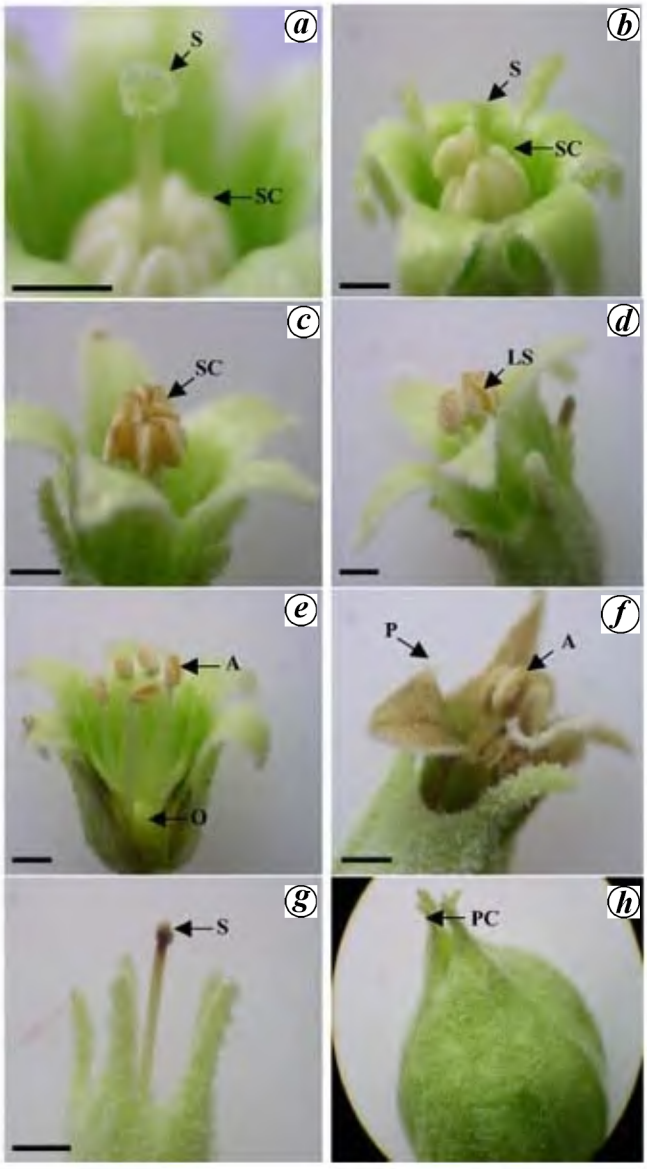

Figure 1. Flower of *Withania somnifera* just after anthesis showing papillate, globose, exserted stigma (S) beyond the staminal cone (SC) (***a***), relative position of receptive stigma and staminal cone after two days of anthesis (***b***), staminal cone connivent about receptive stigma after 3 days of anthesis (anther dehiscence stage) (***c***), arrow-head showing pollen presentation through longitudinal slit (LS) on outer of the stigmatic side (***d***), ovary (O) after fertilization and reflexed-dehisced anthers (A) (***e***), abscising petals (P) and anthers after 6–7 days of anthesis (***f***), wilted stigma (***g***) and persistent inflated conical calyx with pointed and connivent teeth (PC) enveloping fruit, 12–15 days after fertilization (***h***). Bar = 1 mm.

Breeding system

The results in relation to breeding system, fruit and seed set are summarized in Table 3. Pollination in *W. somnifera* is strictly chasmogamous. Flowers exhibit protogynous type of dichogamy as the stigma attains receptivity about 48 h prior to anther dehiscence. The species is self-compatible as demonstrated by manual geitonogamous pollination and spontaneous self-pollination (bagged) treatments. There was absolutely no seed set in emasculated bagged flowers, thus precluding the occurrence of apomixis. Seed set in emasculated open-pollinated and xenogamous cross-pollinated flowers establishes the occurrence of outcrossing in *W. somnifera*. The exclusion of insects in geitonogamous and spontaneous selfing treatments does not preclude fertilization and seed set. Pollination experiments thus amply elucidate that *W. somnifera* under natural conditions is a preferential outbreeder. Mixed mating is encouraged as a consequence of partial temporal dichogamy (protogynous type) as the receptive stigma remains exserted beyond the undehisced staminal cone for about 48 h, thereby facilitating cross-pollination through insect visitation. In case of failure of pollinators to visit individual flowers, autonomous selfing is assured as the filaments elongate and anthers form a cone connivent about the stigma. In this floral configuration when the anthers and stigma are in intimate proximity, anther dehiscence occurs and ensures sufficient pollen deposition on the re-

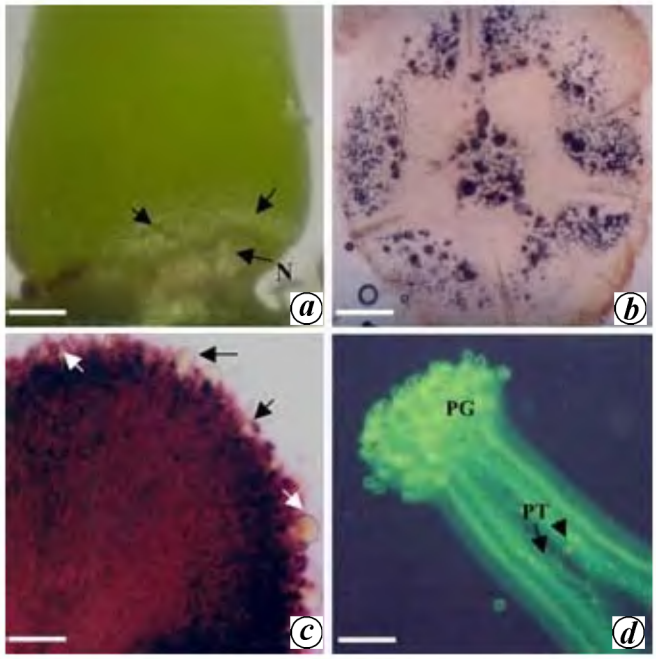

Figure 2. Nectariferous ovary base (N) showing nectar exudates (see arrows) (bar = 295 µm) (***a***), cross-section of nectariferous ovary base revealing nectar accumulation (dark spots) in parenchyma tissue (bar = 286 µm) (***b***), stigmatic receptivity marked by lipiodal secretions all over (bar = 105 µm) (***c***), fluorescence micrograph showing stigma clogged with germinating pollen grains (PG) and part of stylar region with fluorescing pollen tubes (PT) (bar = 242 µm) (***d***).

Table 2. Relative dimensions of flower, pistil and stamens at three developmental stages of flowering of *W. somnifera*

	Anthesis	Stigma receptivity	Anther dehiscence
Flower size* (length × breadth mm)	6.02 ± 0.012 × 3.5 ± 0.016	6.02 ± 0.11 × 3.5 ± 0.17	6.02 ± 0.012 × 3.6 ± 0.17
Pistil length (mm)	4.51 ± 0.11	4.62 ± 0.13	4.76 ± 0.15
Anther size			
Filament length (mm)	1.95 ± 0.03	2.94 ± 0.12	3.84 ± 0.11
Anther lobe length (mm)	1.17 ± 0.03	1.17 ± 0.02	1.18 ± 0.04

*Breadth measured at the top of the corolla.

Table 3. Fruit set and seed set comparisons between natural and hand-pollination treatments in *W. somnifera*

Pollination treatment	Number of flowers used	Per cent fruit set	Mean seed set per flower	Seed set frequency	Per cent seed set	ISI
Intact open-pollinated (control)	272	48.53	25.24 ± 0.54	16–37	76.02	–
Geitonogamy	37	51.35	25.34 ± 0.47	16–38	76.32	1.06
Xenogamy	25	48.00	26.03 ± 0.41	16–37	78.40	–
Spontaneous autogamy	54	50.85	25.02 ± 0.42	16–39	78.37	–
Emasculated open-pollinated	92	27.17*	19.08 ± 2.74	14–37	57.47*	–
Emasculated without pollination	14	0	–	–	–	–

*Difference between intact open-pollinated (control) and emasculated open-pollinated was highly significant at $P < 0.001$ according to 't' test.

ceptive stigma. After anther dehiscence, male and female phases overlap for about 6–10 h. This is followed by cessation of stigmatic receptivity.

Fruit set percentage among different pollination treatments ranged from 27.17 to 51.35%. There was no significant difference in flowers that matured into fruits between hand-pollination treatments (geitonogamy and xenogamy) and control. However, difference in fruit set in emasculated open-pollinated flowers versus intact flowers (control) was significant under natural conditions. Fruit abscission in hand-pollinated and natural-pollination treatments occurred 3–4 days after fertilization. Reduced fruit set observed in case of emasculated open-pollinated flowers was due to both flower and fruit abortion. In this situation about 18% flowers failed to receive pollen due to lack of insect visitation. Pollen load on receptive stigmas under the protogyny ranged from 0 to 312 (mean = 186 ± 23.6; $N = 39$).

The ISI obtained as the ratio of percentage of fruit set through self-pollination (50.85) and cross pollination (48.00) was 1.06, indicating absolute self-compatibility (ISI > 1 indicates self-compatibility)[23].

Seed-set frequencies ranged from 15 to 39. Seed set in geitonogamous and xenogamous pollination treatments was not significantly different from naturally open-pollinated control. However, the difference in ovules that matured into seeds in intact open-pollinated flowers (control) versus emasculated open-pollinated flowers was significant (Table 3).

Male–female effort and pollen–ovule ratio

Pollen production per anther averaged 4195 ± 391.43 and amounted to 20,975 pollen per flower. Pollen grains are tricolpate, slightly sticky, smooth-walled and two-celled at anther dehiscence. They are uniform in size (mean = 43.17 µm ± 0.17). Pollen fertility at shedding stage ranged from 81.30 to 87.63%. Ovules differentiating per flower averaged 33.20 ± 2.45 and ranged between 22 and 45. Pollen–ovule ratio averaged 1.63.

Preemergent reproductive success with open pollination

Reproductive success is divided into two phases: preemergent (PERS, the number of viable seeds that enter the ambient environment), and postemergent (percentage of progeny that survives to reproduce)[24]. We measured PERS with natural, open-pollination flowers during the annual growth cycle. Mean number of inflorescences per plant was 1463 ± 29.42 and number of flowers per cyme averaged 9.01 ± 1.2. Fruit load per cyme averaged 4.5 ± 0.29. Mean number of flowers per plant that matured into fruits was 6525.3 ± 231.47 (~49%). S/O and Fr/Fl ratios were 0.76 and 0.49 respectively. Reproductive success determined (Fr/Fl × S/O) was 0.37 (37%).

Somatic chromosome complement and meiotic system

Diploid chromosome count is 48 (Figure 3 *a* and *b*). Based on chromosome metrics and position of the centromere, the standard karyotype formula for *W. somnifera* can be expressed as: 10 M + 9 SM + 5 ST. Total chromatin length is 47.51 µm; the longest and smallest being 3.04 µm and 1.37 µm respectively. The ratio of the longest to shortest

approaches 2.22. The karyotype is asymmetrical and on the basis of proportion of chromosomes with arm ratio greater than 2 : 1 (= 0.41) and ratio of longest to shortest chromosome (= 2.22), the karyotype falls into '2B' category of Stebinns[30] chart of chromosome asymmetry. Meiosis is normal with perfect chromosome pairing and 24 bivalents at metaphase-I (Figure 3 *d*). Chromosome segregation is regular with 24 : 24 disjunction at anaphase-I (Figure 3 *e*). Chiasmata frequency at diakinesis averaged 47.2 ± 0.71. Recombination index thus averages 71.2.

DNA fingerprinting: Molecular polymorphism

Polymorphic data based on RAPD profile are presented in Table 4. Out of 39 primers, nine arbitrary primers (OPA-14, OPB-03, OPB-10, OPB-13, OPC-08, OPD-12, OPD-20, OPG- 03 and OPH-02) were selected on the basis of scorable amplification products. A total of 149 amplicons were produced by nine primers, of which 130 were polymorphic (87.92%) in open-pollinated progeny. Selfed progeny produced 104 amplicons and 83 bands produced were polymorphic (79.81%). An example of the representative profiles of eight plants from each progeny is shown in Figure 4. Average number of bands per plant ranged from 2 to 9, and the bands amplified ranged in size from 250 to 3478 bp. The average number of bands per primer ranged between 12 (OPH-02) and 23 (OPB-13), with a mean of 16.56 in open-pollinated progeny. In self-pollinated progeny, the average number of bands per primer ranged between 8 (OPH-02) and 19 (OPD-20), with an average of 11.56. The large proportion of exclusive RAPD loci provides a good measure of genetic diversity. This was illustrated by higher average value of Shannon index per primer for open-pollinated progeny (5.71) than self-pollinated progeny (4.68). The genetic diversity values ranged between 3.01–9.36 and 2.76–6.23 for open-pollinated and selfed progeny respectively (Table 4). Marker utility is the function of information content per marker and to characterize the capacity of each marker to reveal polymorphic loci, we calculated PIC. The values for PIC markers ranged between 0.21 and 0.48, with a mean value of 0.313.

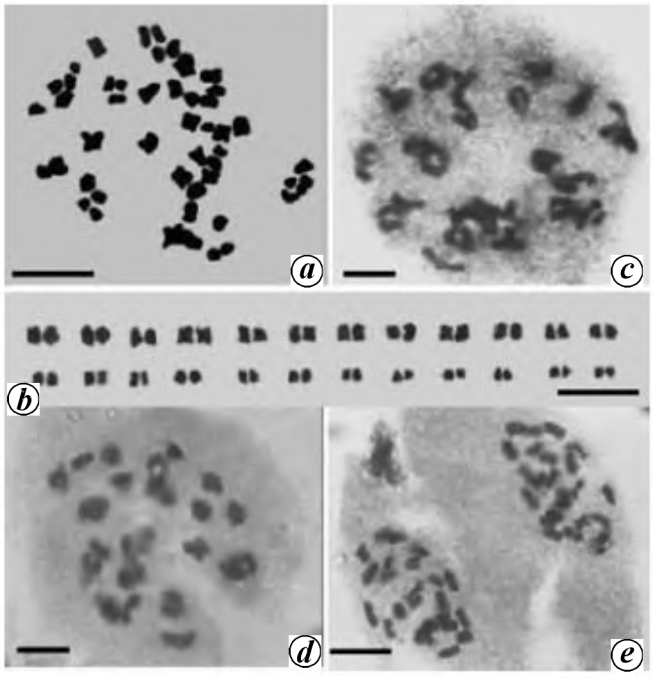

Figure 3. Somatic metaphase spread showing 48 chromosomes (*a*), the karyoidiogram thereof depicting 10 M + 9 SM + 5 ST (*b*), diakinesis (*c*), meiotic metaphase-I presenting 24^{II} (*d*), and late anaphase-I showing normal 24 : 24 disjunction (*e*). Bar = 10 µm.

Discussion

An interesting outcome of the present study is the exposition of the versatility of the mating system presented by self-compatible hermaphroditic flowers of *W. somnifera*. Our results indicate that individual flowers exhibit partial temporal dichogamy of protogynous type, in which stigma becomes receptive prior to anther dehiscence and remains exserted beyond the reach of the staminal cone. Functional dimension and floral configuration expose the receptive stigma to receive cross-pollen through insect vectors. In a probable situation of non-receipt of cross-pollen due to infrequent or unavailability of pollinators, autogamous fertilization is assured by the upward staminal movement as the filaments elongate and anthers form a cone connivent about the stigma. This 'hug me' proximity prompts anthers to dehisce and facilitate transfer of copious pollen autonomously on the receptive stigma. Thereafter, overlap of male and female phases lasts for 6–10 h. The whole mechanics of the mating process thus results in mixed mating in which the individual flowers are either open-pollinated or self-pollinated and the plant produces a mixture of self and outcross seed. Pronounced protogyny provides for strong outcrossing. Floral phenoevents suggest that selfing and outcrossing are mutually exclusive and non-competing as the autonomous pollen arrives late during floral ontogeny. We view dichogamy of protogynous type more adaptive and advantageous than protandry in mixed mating plants. Protogyny would favour delayed selfing and consequently reduce pollen discounting as more pollen would be made available for outcrossing.

Results from pollinator exclusion treatment amply demonstrate that autogamous reproduction is able to provide reproductive assurance (S/O = 0.76) in the absence of pollinator visitation. Fruit and seed set did not differ significantly between autogamous and xenogamous pollination treatments (Table 3), indicating no seed discounting or any influence on fruiting success. Comparison between intact open-pollinated versus emasculated open-pollinated flowers showed significant difference in seed-set efficiency and fruiting success, implying pollinator inconsistency

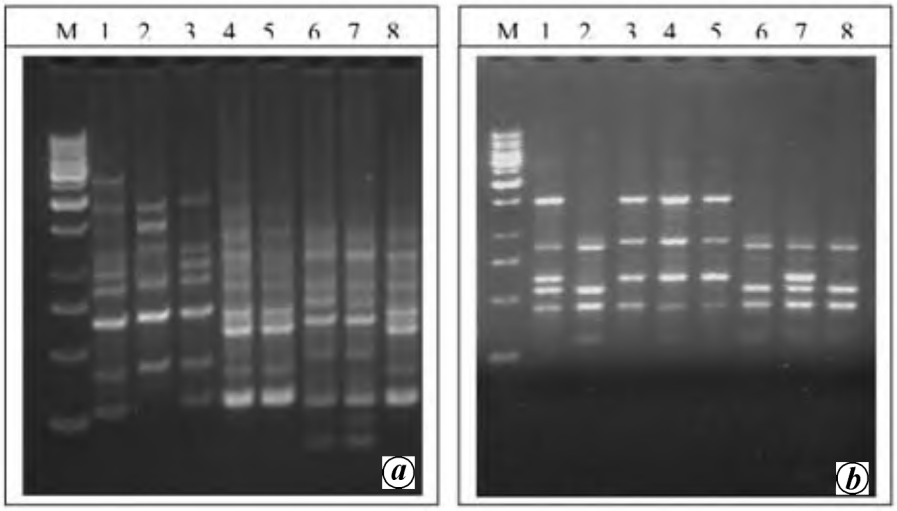

Figure 4. DNA fingerprints obtained with primer OPB-13. ***a***, Lanes 1–8, Open-pollinated progeny. ***b***, Lanes 1–8, Selfed progeny. M, Molecular weight marker (10 kb DNA ladder).

Table 4. Primer-wise proportion of polymorphic products generated through PCR amplification and comparison of genetic diversity in open and self-pollinated progenies of *W. somnifera*

Primer number	Primer sequence (5′ → 3′)	Total no. of bands		Per cent polymorphism		Shannon index		PIC
		OP*	S**	OP	S	OP	S	
OPA-14	TCTGTGCTGG	16	9	75.00	77.78	9.36	6.23	0.48
OPB-03	CATCCCCCTG	23	16	95.65	68.75	6.04	5.08	0.37
OPB-10	CTGCTGGCAC	14	12	92.86	91.67	3.50	3.62	0.22
OPB-13	TTCCCCCGCT	17	9	64.71	66.67	5.12	4.98	0.27
OPC-08	TGGACCGGTG	15	11	93.33	81.82	3.01	2.76	0.29
OPD-12	CACCGTATCC	17	11	94.12	90.91	4.96	4.03	0.30
OPD-20	ACCCGGTCAC	21	19	90.48	73.68	7.10	6.50	0.21
OPG-03	GAGCCCTCCA	14	9	100.00	88.89	5.06	3.78	0.26
OPH-02	TCGGACGTGA	12	8	83.33	75.00	7.24	5.12	0.42
Total score		149	104	–	–	51.39	42.1	2.82
Mean per primer		16.56	11.56	87.92	79.81	5.71	4.68	0.313

*Open and **Self-pollinated progenies.

and pollen limitation under female phase. Nevertheless, in open pollination it is difficult to distinguish between geitonogamous and cross-pollen as the ovules in the present case, during female phase may be sired either by cross-pollen, geitonogamous pollen or geitonogamous plus cross-pollen. A breeding system that promotes cross-pollination (thereby facilitating genetic variability) while maintaining the capacity to self-pollinate is probably adaptive in changing environment where pollinators may be unreliable. As contended by many authors the benefit of reproductive assurance provided by autonomous selfing may be 'context-dependent as the pollinators are notoriously variable in space and time due to fluctuations in abiotic factors and variation in seasonal patterns of abundance'[31–33].

The floral nectary is a conspicuous feature of many plant families[34]. Here the floral nectary is present at the ovary base and continues nectar production from late-bud stage (S_2) until fertilization (S_6) (Table 1). However, it is more active during stigmatic receptivity. Nectar presentation in *W. somnifera* offers greater chance of attracting pollinators under female phase. Copious nectar production is an adaptation to facilitate xenogamy[35].

The species practices stiff pollen competition (pollen load = 924.64 ± 28.43; $N = 10$) during autonomous selfing (male–female phase) as there are about 28 pollen grains available to sire one seed. Comparable seed set in geitonogamous, xenogamous and open-pollination treatments with no significant differences suggests that there is no uncertainty factor about pollen genotypes (self/cross) landing on the stigma. This is in context to the prediction that outcrossing species show greater seed abortion than self-pollinating species[36,37].

High P/O ratio (1.63) is in conformity with the predictions for xenogamous species[21], whereas fairly high S/O ratio (0.76) is in agreement with the postulates for autogamous species[38]. It has been argued that male fitness is achieved through excessive pollen donation (here, 4195 ± 391.43 per anther) and species with larger number of

ovules (in this case 32.20 ± 2.45 and frequency 22–45) per flower invest more in male function than plants with fewer ovules per flower, and the greater investment is expressed as a larger number of pollen grains[39]. An interesting floral adaptation to avoid pollen discounting was displayed by the mechanics of anther dehiscence. Anthers dehisce through longitudinal stromial slits on either of the stigmatic sides (Figure 1 *d*), thus presenting more pollen to the foragers for food and export.

PERS determined was 37% and in a roundabout assessment indicates an average of outcrossing perennials and inbreeding annuals[38]. PERS gives the measure of total percentage of ovules maturing into seeds (fecundity). Fr/Fl ratio was 0.49. One of the many explanations proposed to account for abundance of flowers is that 'excess' flowers are borne simply to fulfil male function and fruits are aborted to adjust the number to available resources[36,40,41]. Excess of flowers would thus ensure overall fitness (male and female). Nevertheless, in the present case PERS under cultivated conditions and natural habitats is likely to vary significantly due to fluctuations in resource availability and ambient environment.

An important feature of the genetic system of *W. somnifera* seems to be high chromosome number ($2n = 48$). Total DNA content (chromatin length = 47.51 μm) is distributed asymmetrically ('2B' category of Stebbins) among chromosomes. High recombination index (71.2) coupled with mixed mating are indications towards adequate genetic variation and wide genetic base. Both the breeding system as well the meiotic system promise to generate sufficient variation in the species.

In the present study, RAPD assay revealed higher proportion of polymorphic loci (87.92%) in open-pollinated progeny in comparison to selfed (79.81%). Genetic diversity based on Shannon's index averaged 5.71 and 4.68 for open-pollinated and self-pollinated progenies respectively. Understandably open-pollinated progeny would tend to be more heterozygous on account of higher proportion of polymorphic loci. However, diversity values do not indicate substantial differences between the two progenies and seem to be congruent with the breeding behaviour of the species. Sexual system of mixed mating would tend to maintain balanced polymorphism or intermediate levels of heterozygosity through both autogamous as well as allogamous components of sexuality. Autogamous component would ensure release of variability through meiotic recombination and transmission of genes through self-pollen only, whereas allogamy would guarantee sufficient variability through random fertilization (outcrossed pollen and ovules) as well as meiotic recombination.

W. somnifera thus exhibits a blend of features that are in conformity with its breeding behaviour. Perenniality, strong protogyny, copious pollen production, pollen of uniform size, availability of nectar, high P/O ratio and inflorescences well above the ground account for its outcrossing nature. Small flower size, high Fr/Fl and S/O ratios are some of the features associated with autogamous species. Mixed mating presents an opportunity to receive potential benefits from outcrossing as well as selfing while lessening the impact of pollinator limitation[42]. Species with floral development mechanisms that promote outcrossing when pollinators are present, but ensure self-pollination if they are not[43], guarantee reproductive assurance and provide a 'best of both the worlds' scenario[31,33,44].

It would be conceptually interesting to relate the possible implications of reproductive strategy adopted by the species to the chemotypic/bioactive variability and in the ecology and plant–herbivore interactions. In some crosses involving different chemotypes, hybrid chemoprofiling has revealed a complex pattern of inheritance of various constituents. A snapshot of the literature[45–49] reveals that in hybrids, the suite of chemicals may be (i) similar to that in one of the two parents, (ii) intermediate between the two parents, (iii) present in higher concentrations, or (iv) in lower concentrations than in either parent, and (v) novel constituents that are lacking in both the parents. The rationale for elucidating the literature pertaining to hybrid chemistry here is to emphasize the implications for the hitherto unidentified biochemical pathway for chemical transformation of steroidal lactones (withanolides) via the sexual system – gene interactions and inheritance patterns.

It is well known that hybridization results in progeny that differ qualitatively and quantitatively from parents in expression of secondary metabolites[50]. Several studies have shown that plant secondary chemistry alters resistance of hybrid plants[51] to herbivores. The promise of sexual reproductive success and preferential outcrossing nature of *W. somnifera* may be viewed as an important evolutionary mechanism for generation and maintenance of sufficient chemical polymorphism in relation to plant–herbivore interactions and nonrandom survival of progenies under marginal and xeric habitats.

Both theoretically and empirically, manipulative hybridization has implications for production of novel bioactive molecules and prospects for qualitative and quantitative amelioration as the species displays an appreciable variability and an efficient genetic system.

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