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Full Length Research Paper

Reproductive behaviour and breeding system of wild and cultivated types of *Withania somnifera* (L.) Dunal

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The Indian germplasm of Withania somnifera (L.) Dunal shows remarkable genetic variability both in the cultivated and the wild populations. The utilization of this variability through conventional breeding requires a clear understanding of its reproductive biology and breeding system. It is an amphimictic species practicing open pollination. However, a proximal placement of the stigma and the anther and a synchrony between the receptivity and dehiscence of anthers strongly predispose the species to self pollination and selfing. However open pollination results in equally high percentage of fruit and seed set as on controlled selfing indicating that the species shows facultative autogamy. This raises the probability of genetic improvement through hybridization. The somatic complements revealed a diploid number of 2n=48 in all the accessions tested. The absence of karyomorphological differences indicated that numerical and structural changes do not have a role in controlling the genetic variability of the species. Experimental crosses between the cultivated and the wild accessions produced viable seeds. A significantly higher fruit set and seed germ inability in crosses involving the cultivated types as the seed parent point to the existence of maternal effect.

Key words: *Withania somnifera*, floral biology, autogamy, geitonogamy, open pollination, ovule ratio and reproductive effort.

INTRODUCTION

Withania somnifera (L.) Dunal (Ashwagandha, Solanaceae), popularly known as Indian ginseng and Winter cherry, is one of the most reputed medicinal herbs that forms an essential constituent of over 100 traditional medicine formulations (Kaileh et al., 2007). The well described pharmacological activities of the plant include physiological and metabolic restoration, anti-arthritic, anti-aging, anti-cancer, anti-bacterial, cognitive function improvement in geriatric states and recovery from neurodegenerative disorders (Owais et al., 2005; Lal et al., 2006; Misra et al., 2008; Mirjalili et al., 2009; Koduru et al., 2010; Ven Murthy et al., 2010). *In vitro* and *in vivo* pharmacological investigations have elucidated the association of these activities to withanolides present in leaves and roots (Sangwan et al., 2004; Mirjalili et al., 2009). The species grows wild in several states of India viz., Madhya Pradesh, Uttar Pradesh, Andhra Pradesh, Gujarat, Maharashtra, Rajasthan and Punjab extending to the mountainous regions of Himachal Pradesh and Jammu up to an elevation of 1500 m (Singh and Kumar, 1998). In view of increasing demand in phytopharmaceutical industries, an annual morphotype has been in cultivation in Madhya Pradesh, Rajasthan and Andhra Pradesh (Kothari et al., 2003). The cultivated plants differ from the wild ones not only in their therapeutic properties but also in morphological characters of roots, stems, leaves, flowers, seeds and fruits. For this reason, Kaul (1957) suggested a new species *Withania ashwagandha* for the cultivated plants as distinct from *Withania somnifera* for the wild ones.

In recent literature, Kumar et al. (2007) reported that

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wild and cultivated populations of Ashwagandha differ not only in morphology but also in their chemical architecture and suggested that the wild and the cultivated populations be recognized as separate chemotypes. Recently our group reported the differences in molecular make up of the wild and the cultivated populations (Mir et al., 2010; Kumar et al., 2011). Joshi et al. (2010) also confirmed the distinct nature of wild and cultivated plants on a number of morphological, bio-chemical and physiological traits. Moreover, protocols of breeding require a thorough understanding of the reproductive behavior and crossability of the morphotypes. In view of paucity of such data on the Indian chemotypes, the present investigations were conducted to fill this gap of information. Our work is a sequel to integrated investigations on the Indian germplasm of *W. somnifera* under the New Millennium Indian Technology Leadership Initiative (NMITLI) programme. We took leads from the initial work of Kaul et al. (2005) who studied some of the aspects of the reproductive biology of *W. somnifera*. Reference to the work on the related species *Withania aristata* by Anderson et al. (2006) has also proved helpful.

MATERIALS AND METHODS

Plant material

Data were collected from populations of twenty three wild and cultivated accessions of *W. somnifera* maintained in the experimental plots of Indian Institute of Integrative Medicine, Jammu, India. For comparison, two elite accessions (AGB002 and AGB025) were taken as representative of the wild and cultivated accessions, respectively. The site of study is located at 32^0 44' N, 75^0 55' E. It is 400 m asl with the annual temperature ranging between 5 and 45°C, and annual rainfall up to 110 cm. Studies were carried out during the peak flowering period (April to June) for three consecutive years (2007 to 2009).

Floral biology

Individuals in both the populations (wild and cultivated) bore perfect flowers. Morphology and morphometry was done from mature flowers. Flowering phenology was studied on day to day basis from twenty plants marked in each population at the pre-anthesis and post-anthesis phases.

Pollen/ovule measurement

Anthers collected from twenty fully mature flowers at pre-anthesis stage were stored in 1% glycine. The anthers were chopped, spun on vortex mixer and pollen grains were counted with a haemotocytometer (Osankoyo, 1999). Pollen size was recorded with the help of a set of ocular and stage micrometers. Pollen output per flower was calculated by the estimated number of pollen grains per anther times the number of anthers (5) per flower. Ovule count per ovary was made by clearing fresh pistils with 1 N NaOH, staining with 1% aceto-carmine and gentle squashing. Ovules were counted under a stereo-microscope. Pollen-ovule ratio was obtained by dividing the pollen number by the number of ovules per flower (Cruden, 1977). Pollen viability was estimated by 3, 5-triphenyltetrazolium chloride (TTC 2,) (Stanley et al., 1974) and Fluorescein Diacetate (Heslop-Harrison and Heslop-Harrison, 1970) tests. For ultra-structure of the pollen surface, pollen was acetolyzed and mounted on glass plate following the method of Caccavari and Dome (2000). Thereafter, plates were prepared for electro-micrographs under a Scanning Electron Microscope (JEM-JEOL-100 CX II EM with ASID) at 40 KV.

Pollination

Stigma receptivity was checked by the method of **Shivana** and Rangaswamy (1992). Frequency of flower visitors was recorded at regular intervals of time during the peak flowering period between 9.30 am and 5.00 pm for three consecutive days.

Reproductive effort

Dry biomass was estimated from ten plants each from wild (AGB002) and cultivated (AGB025) populations. The plants were disjointed into leaves, roots, flowers and fruits. Leaf area was measured by leaf area meter and seed weight with a single pan electronic balance. Foliar, floral and reproductive shoot parts were oven dried at 60°C to a constant weight. Reproductive effort (RE) was estimated by three indices.

$$RE_1 = \frac{GW}{GW + LB}$$ (Wolff, 1999)

$$RE_2 = \frac{FB}{LB + GW}$$ (Dunn and Sharitz, 1991)

$$RE_3 = \frac{SW}{LA}$$ (Primack and Antonovics, 1982)

Where, GW, generative weight (Floral biomass plant^{-1}+ Reproductive shoot biomass plant^{-1}); LB, leaf biomass plant^{-1}; FB, floral biomass plant^{-1}; SW, seed weight (total) plant^{-1}; and LA = leaf area plant^{-1}.

Breeding experiments

Controlled crosses were carried out to test the wild and cultivated populations for autogamy, geitnogamy and xenogamy. Hand pollinations were conducted during 0900 to 1100 h during April to May for three consecutive years (2007 to 2009).

Autogamy

In a sample, twenty plants flowers were emasculated before anther dehiscence and pollinated during peak stigmatic receptivity with pollen from previous day flowers and bagged. Self-pollination (passive autogamy) was tested by bagging intact flowers.

Geitonogamy

Hand pollination of 155 flowers was conducted in twenty plants each of the wild and the cultivated population with pollen from other

flowers of the same plant.

Xenogamy

Here again, twenty plants each from wild and cultivated populations were involved in experiments to test for xenogamy. An inflorescence from each of the selected plants was marked and open flowers were removed. Flower buds ready to open were carefully emasculated avoiding damage to the pistil. For hand pollination, mature pollen from the male parent were put on receptive stigma of the emasculated flowers (n=300) with the help of a paint brush and bagged. After time for fruit set elapsed, bags were opened and percentage fruit set and number of seeds/fruit was recorded. At fruit maturity, seeds from different crossing experiments were dried and put in Petri plates at 25°C in controlled conditions for germination; percentage germination was recorded after 30 days.

Somatic complements

Seeds from the parent populations and those raised by hybridization were germinated in the dark at 27°C on moist filter paper in Petri-dishes. Root tips ~ 0.5 cm long were excised pre-treated in a saturated solution of *p*-dichlorobenzene (PDB) for 4 h and rinsed thoroughly 5 to 6 times with double distilled water. Then they were fixed in acetic: ethanol (1:3) mixture for 24 h and stored at 4°C in 70% ethanol. For chromosomal studies, fixed root tips were washed thoroughly in distilled water to remove the traces of ethanol. Root tips were then hydrolyzed in 5N HCl for 30 min at room temperature, rinsed thoroughly in distilled water and kept in leucobasic fuschin for 45 min in dark conditions and squashed in 1% acetocarmine. Some of the cells showed well spread metaphase complements.

Chemical profiling

The leaf and root samples for each accession were collected from twenty plants at the same stage of development. They were dried at 45 to 50°C in oven for 48 to 50 h. Dried and powdered roots and leaves were percolated separately four times with ethanol: water (1:1) at retention time (RT). Extracts were centrifuged and concentrated to $1/8^{th}$ of the original volume under reduced pressure at 50±5°C. The concentrated extract was thoroughly extracted with chloroform. Thereafter, chloroform was distilled off under reduced pressure to yield a residue. Qualitative analysis of leaf and root residue was carried out for two withanolides (withaferin A and withanolide A) by High-performance liquid chromatography (HPLC) method according to the procedure of Khajuria et al. (2004).

RESULTS

Plant habit and morphology

Plants of the wild population (AGB002) were perennial shrubs, 120 to 130 cm tall, having sharply acute, slightly haired membranous leaves with entire margins. The leaf blade was 7.0 to 8.5 cm × 4.5 to 5.5 cm in size. Six to seven shoots arose from the crown. Flowers were borne in clusters of 6 to 10 at nodes in the axils of leaves. The flowers were pentamerous, actinomorphic and perfect with a hypogynous ovary. Androecium composed of five epipetalous stamens. The ovary was bilocular with axile placentation and an exserted pistil. The fruiting calyx was globular and very faintly ribbed. The berries were red and the seeds oily to touch. Biochemically, withaferin A was present in significantly higher concentration in the leaves (0.423 to 0.679 g 100 g^{-1}) than in the roots (0.003 to 0.010 g 100 g^{-1}). The amount of withanolide A ranged from 0.001 to 0.003 g 100g^{-1} in the leaves and 0.100 to 0.300 g 100g^{-1} in the roots. The third withanolide, namely withanone also varied from 0.008 to 0.170 g 100 g^{-1} in leaves and 0.011 to 0.017 g 100 g^{-1} in the roots. Plants of cultivated population yield the Ashwagandha of commerce. Plants were much shorter than the wild types (rarely more than 40 cm tall) having small ovate, sub acute, stellately pubescent and subcoriacious leaves with inconspicuous veins and undulate margin. There were no differences from the wild types either in the size or structure of the essential and the accessory whorls (Table 1). However, the fruiting calyx was a little more elongated, and prominently ribbed, enclosed yellow/orange berries with non-oily and dry seeds (Figure 1). The species exhibits perpetual flowering with a peak period in April to June. Withanolide A and withanone were absent in the leaves but withaferin A showed a higher range (0.850 to 1.255 g 100 g^{-1}). In the roots, all the three marker compounds were present in lower quantities.

Floral biology and breeding system

The opening of petal lobes at the tip marked the beginning of anthesis which occurs between 0090 to 1100 h. At pre-anthesis phase, the carpel was longer than stamens but post-anthesis stamens increased in length exceeding the height of the carpels at the time of anther dehiscence. The filaments were inserted into stapets or stirrups which were fused with the corolla tube. In both the populations the stapet (filament base) was adpressed to the ovary and a narrow groove between the stapets and the ovary allowed nectar to ooze upwards from the nectary located at the base of the ovary. After anthesis, the petals lasted for about three days after which the corolla, which was pale greenish at anthesis turned brown and withered. By the next day, the petals dropped. Anthers dehisced longitudinally. On the inner side, they were in touch with stigma causing sufficient pollen deposition on it. Pollen grains were yellow, smooth walled, slightly sticky, uniform in size and nearly as long as broad both in the wild and cultivated types (Table 1). Stigmas were papillose and greenish and had a wet surface at the receptive stage. In both the populations pollen grains were found to be trizonocolporate with a scabrate-granulate exine pattern. The non-viable pollen grains were deformed (Figure 2). In both the

Table 1. Comparison of essential traits in the floral biology of wild and cultivated populations of *W. somnifera.*

Character (Flower size)	Wild population	Cultivated population
(Length × Breadth) mm		
Before antesis	5.2±0.015×5.3±0.027(75) *	5.03±0.022×4.50±0.022
After anthesis	5.34±0.020×5.3±0.026(75)	5.33±0.018×5.23±0.020
Pistil length (mm)		
Before anthesis	3.87±0.011	3.69±0.006
After anthesis	4.52±0.011	3.76±0.006
Length of stamen before		
Anthesis (mm)	3.09±0.06 (75)	3.48±0.13 (75)
Length of stamen after		
Anthesis (mm)	3.76±0.05 (75)	3.75±0.09 (75)
Pollen anther^{-1}	5800±175.7 (20) *	7650±201.5 (20)
Pollen flower^{-1}	29000	38250
Pollen viability		
TTC test	66.37±1.84 (2100)	62.4±1.13 (2320)
FDA test	60.50±0.85 (2100)	50.06±7.57(2320)
Pollen shape	Spherical to sub-spherical	Slightly triangular
Pollen size (µm)	28.0±0.07	27.3±0.07
Stigma type	Pappilose wet type	Pappilose wet type
No. of ovules flower^{-1}	35.5±1.85 (20)	31.5±1.20 (33)
Pollen ovule ratio flower^{-1}	817:1	1214:1

* Mean ± Standard Error; Values in parenthesis represent number of observations.

representative accessions (AGB002 and AGB025), berries took 30 to 40 days to mature. Both the wild and the cultivated types as also their synthetic hybrids had a somatic number (2n) = 48. They did not show any karyomorphological differences (Figure 2 A to B). Essential data on the reproductive morphometry and pollen and ovule output are summarized in Table 1.

Experimental crosses

Manual pollination of flowers with self pollen resulted in 82.5± 2.36 and 76.80±1.68% fruit set in wild and cultivated populations, respectively (Table 2). In the wild population, of the total fruit set, 83.0± 2.89% developed mature seeds and out of the total mature seeds 60.3±4.57% showed germination. However in the cultivated plants, seed set and germination percentage were 74.5±1.28 and 58.3±4.41, respectively (Table 2). Percentage fruit and seed set as also percentage germination on geitnogamy were found to be in the same range as for autogamy and open pollination (Table 2). Artificial crossing between plants of wild and cultivated populations led to very low fruit set percentage 4.04±3.21 (for W♀ X C♂) and 8.3±2.71 (for C♀ X W♂) (Table 2). The seeds obtained as a result of crossing showed a germination percentage of 10.8±4.93 in direct (W♀ X C♂) and 20.3±5.15 in reciprocal (C♀ X W♂) crosses, respectively. In crossing experiments higher figures for fruit set, seed set as well as germination percentage were obtained when wild plant was the source of pollen.

Reproductive effort

Dry biomass of foliar and floral parts exhibited striking differences between the two populations. Total leaf biomass and floral biomass per plant averaged to 21.35 and 13.40 g, respectively in the wild as compared to only3.51 and 11.01 g in the cultivated population (Table 2). The RE_1 was a little lower in wild population (81.26%) as compared to the cultivated (93.31%) types. However the second estimator of RE_2 revealed much lower figures (11.75%) for wild population as compared to the cultivated (24.52%). And the third estimator of RE_3 yielded comparable figures in the cultivated (9.94 mg cm^{-2}) and the wild (8.98 mg cm^{-2}) populations (Table 3).

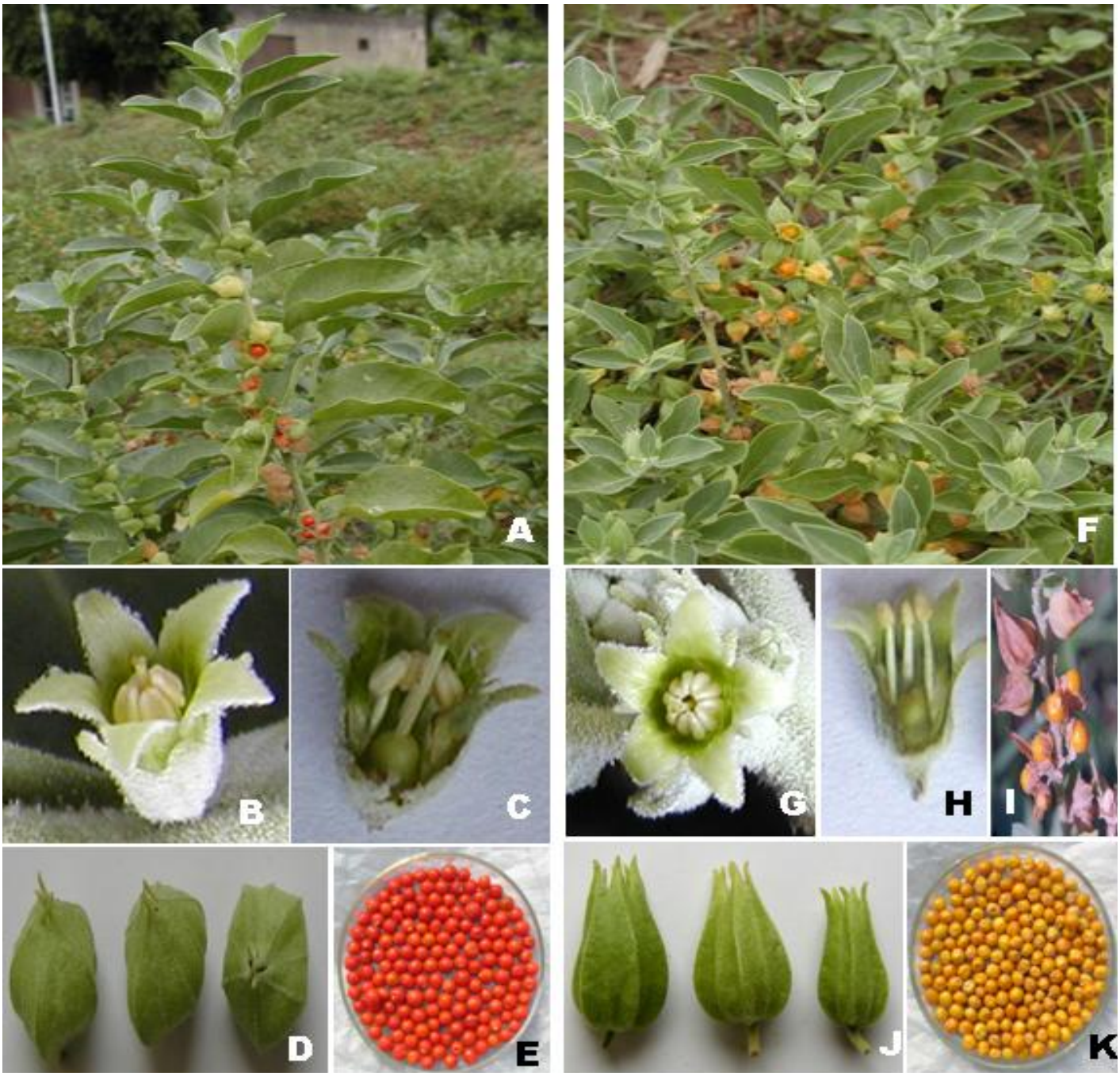

Figure 1. Comparative morphology of wild; A to E and cultivated populations; F to K of *W. somnifera*: Habit; A, F, Flower at anthesis; B, G, L.S. of flower at anthesis; C, H, Inflated calyx; D, J, Mature berry; E, K and (I) Fruit of cultivated type exposed to show attachment.

Insect visits

Both the populations bore scentless and unattractive light green flowers. Nevertheless, they were visited by *Apis dorsata*, *Apis florae*, butterflies and some other insect species. Insect visits commenced early in the morning with a peak activity from 0800 to 1200 h.

DISCUSSION

Flowering plants possess a wide array of contrivances controlling breeding system (Eckert and Barrett, 1994; Gituru et al., 2002). Breeding systems range from obligate selfing to obligate out-crossing and are controlled by various types of self compatible and self

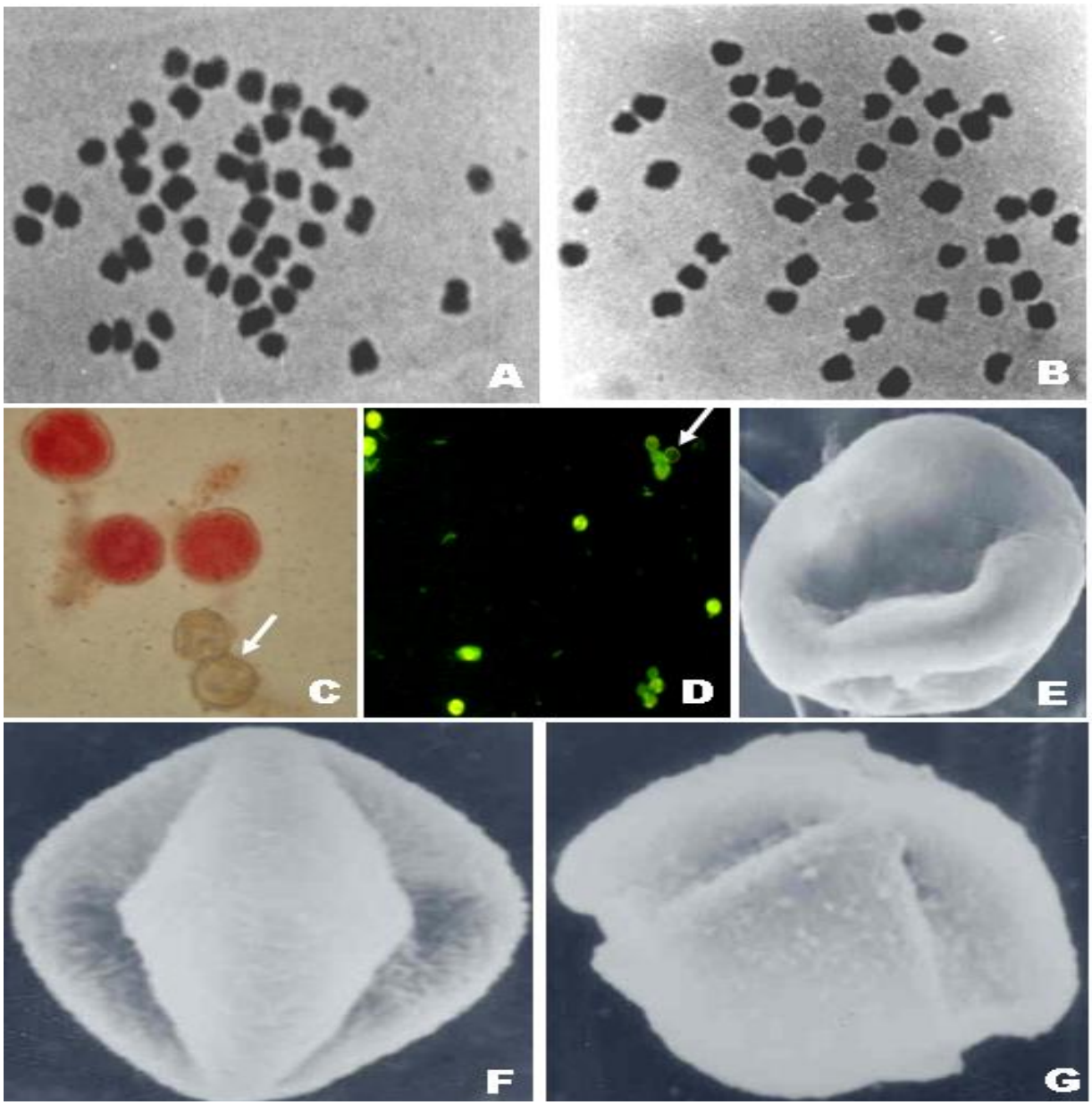

Figure 2. Mitotic chromosome complements of wild; A and cultivated; B plants; pollen viability by TTC; C and FDA; D tests (arrows indicate non-viable pollen); scanning electron micrographs of non-viable; E and viable pollen grains in anterior; F and posterior; G views.

incompatible systems which are often accompanied by peculiarities in floral architecture (Darlington, 1958; Lewis and John, 1963; Bawa et al., 1985; Ramirez and Brito, 1990; Talavera et al., 1993; Lalonde and Roitberg, 1994). Even though a lot of work on reproductive biology and breeding system of medicinal plants has been reported, work on sex expression and reproductive behaviour of *W. somnifera* is scanty. The present studies indicate autogamous nature of the species. Apart from a monoecious type of sex expression, there are other indicators of autogamy in the material under investigation. Equal length of stamens and carpels, close proximity of anthers and stigma and synchrony between stigma receptivity and anther dehiscence strongly predispose this species to self-pollination and autogamy. A similar mechanism of self pollination caused by reflexing of stigma to contact its own anthers has been reported in *Petrocoptis viscose*, an endemic herb of the North-West Iberian Peninsula (Navarro and Guitian, 2002).

Table 2. Pollination type, percentage fruit set and seed germination in wild and cultivated populations of *W. somnifera.*

S/N	Pollination	Population	Fruit set (%)	Seed set/berry (%)	Germination (%)
1	Autogamy	Wild	82.5±2.36* (n=400)	83.0±2.89	60.3±4.57
		Cultivated	(n=273) 76.8±1.68	74.5±1.28	58.3±4.41
2	Geitnogamy	Wild	70.8±4.37 (n=300)	81.8±3.16	55.3±1.41
		Cultivated	60.5±3.27 (n=203)	60.5±4.91	53.2±2.23
3	Controlled crossing (Xenogamy)	♀W×♂C	4.04±3.21 (n=312)	38.5±4.03	10.8±4.93
		♀C×♂W	8.3±2.71 (n=173)	40.3±3.37	20.3±5.15
4	Open pollination (Control)	Wild	87.3±2.28 (n=365)	87.3±3.08	58.10±1.60
		Cultivated	79.40±1.50 (n=215)	85.5±1018	63.0±2.68

***** Mean ± Standard Error; (n) = number of flowers scored; W-wild, C-cultivated.

In addition to proximal placement of stigmas and the anthers, high pollen load both in the wild (817) and the cultivated (1214) per ovule checks competition from foreign pollen and greatly reduces the possibility of cross pollination. Dichogamy at the floral level is an important mechanism controlling out-crossing in a large number (more than 75%) of bisexual angiospermic species (Bertin and Newmann, 1993; Griffin et al., 2000). However in our material, perfect synchrony in the male and the female phases favour self pollination. Also unattractive flowers of the species reduce the possible contribution of insect visitors in pollination and pre-dispose the species to self pollination. This is a frequent feature reported in other self pollinating plants as well (Primack, 1985). Despite the existence of several pointers to the predominance of autogamy, crossing experiments provided the clinching evidence on the breeding system of the species. Most significantly, a high percentage of fruit and seed set in controlled self pollination in both wild and cultivated accessions confirmed their self compatible nature. Cross pollination after emasculation resulted in very low fruit set indicating partial fixation of autogamy in the species (Table 1). Kaul et al. (2005) also reported self compatibility in some other accessions of the species. That *W. somnifera* is autogamous both at the level of the flower and the individual was indicated by our experimental work. Both the cultivated and the wild accessions showed nearly as much percentage seed set on geitonogamy as on autogamy (Table 2).

The difference in fruit set on autogamy and open pollination was also found to be insignificant providing strong evidence that the species is cross compatible at the population level and raises the possibility of inter-populational crossability between the wild and the cultivated types. However, fruit set, seed set and seed germinability of the hybrid seed by controlled crosses between the wild and the cultivated types was lower than that obtained on autogamy and open pollination by several orders of magnitude (Table 2). However figures for these parameters of crossability were significantly higher when the cultivated type were used as the seed parent indicating some kind of a maternal effect. Comparatively lower fruit and seed set in the inter-population crosses points to genetic divergence between cultivated and wild accessions and lend support to the proposal that cultivated types be put into a separate species *W. ashwagandha* (Kaul, 1957). All along the period of cultivation, the cultivated types seem to have accumulated genetic differences from the wild; autogamy seems to provide a means for this fixation and consequent divergence.

Resource allocation constitutes an important aspect of the life cycle of plants (Primack and Antonomvics, 1982; Dunn and Sharitz, 1991). Higher reproductive effort as estimated by three indices (RE_1, RE_2 and RE_3) in the cultivated type is another result of domestication (Table 3). Similar differences were reported in several species of the plantain genus *Plantago*. Cultivated species, *Plantago ovata* invests more on reproductive effort as compared to its wild allies- *Plantago major* and *Plantago lanceolata* (Sharma and Kaul, 1995). Despite genetic divergence, crossability of the types provides a channel for genetic exchange and improvement. Even earlier, crosses involving different chemotypes have been attempted for genetic improvement of the species. The biochemical profiles in the hybrids may be similar to one of the two parents, intermediate between them and even show novel constituents that are lacking in both the parents (Kirson et al., 1977). For example, inter-chemotypic hybrids between Israel chemotype III and Indian chemotype I produced an appreciable amount of withanolide D which was absent in both the parental types (Kirson et al., 1977; Singh and Kumar, 1998).

Table 3. Biomass allocation and reproductive effort in wild and cultivated populations

Character	Wild population	Cultivated population
No. of leaves plant^{-1}	152.50±16.01*	70.25±10.28
Dry biomass leaf^{-1} (g)	0.14±0.004	0.05±0.005
Dry leaf biomass plant^{-1} (g)	21.35	3.51
Floral biomass plant^{-1} (g)	13.40±2.31	11.01±2.24
Rep. Shoot biomass plant^{-1} (g)	79.21±6.24	30.38±2.26
Generative weight (g)	92.61	41.39
Reproductive effort (RE_1)	81.26%	93.31%
Reproductive effort (RE_2)	11.75%	24.52%
Total leaf area plant^{-1} (cm^2)	2671.80	635.76
Total seed weight plant^{-1} (g)	24.00±3.12	6.32±0.62
Reproductive effort (RE_3) mgcm^{-2} (Seed wt. /unit leaf area)	8.98 mg/cm^2	9.94 mgcm^{-2}

* Mean ± Standard Error.

Chemotypic profiles of some other inter chemotypic hybrids in *W. somnifera* have also been worked out (Lavie et al., 1975; Nittala and Lavie, 1981; Eastwood et al., 1980).

Conclusion

W. somnifera is an open pollinated species. However, floral architecture in both the cultivated and the wild populations creates condition for selfing. The divergence seen between the cultivated and the wild accessions could probably have resulted from genetic fixation through selfing. However formation of fertile hybrids in interpopulation crosses indicates that the genetic barriers are partial. Synchrony in the flowering periods of the wild and cultivated accessions, monoecious sex expression and an amenability to emasculation and crossing further enhance the possibility of genetic improvement of this amphimictic species through hybridization.

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