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An Overview on *Withania somnifera* (L.) Dunal – The 'Indian ginseng'

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ABSTRACT

Withania somnifera (L.) Dunal (Family: Solanaceae; commonly known as Ashwagandha; English name, winter cherry) is a perennial plant species with profound therapeutic significance in both traditional (Ayurvedic, Unani, Sidhdha) and modern systems of medicine. Due to the restorative property of roots, the species is also known as 'Indian ginseng'. With a view to the medicinal importance of the species an overview is conducted involving nearly all essential aspects to provide updated, adequate information to researchers for effective utilization in human benefit.

Keywords: Ashwagandha, Ayurveda, Sidhdha, Solanaceae, Unani

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; **A1**, anaphase-1; **AFLP**, amplified fragment length polymorphism; **ARS**, Agricultural Research Service; **b.i.d.**, bis in die; **B_5**, Gamborg's medium; **BA**, 6-benzyladenine; **BAP**, 6-benzylaminopurine; **CD**, cyclin dependent; **cDNA**, complementary DNA; **CIMAP**, Central Institute for Medical and Aromatic Plants; **dES**, diethyl sulphate; **DF**, degree of freedom; **DW**, dry weight; **EMS**, ethyl methane sulphonate; **EST**, expressed sequence tag; **FYM**, farm yard manure; **GA_3**, gibberellic acid; **GCV**, genotypic coefficient of variation; **GO**, gene ontology; **HA**, hydroxyl amine; **HPLC**, high performance liquid chromatography; **HPLC-DAD**, high performance liquid chromatography-diode array detector; **HPTLC**, high performance thin layer chromatography; **IAA**, indole-3-acetic acid; **IBA**, indole-3-butyric acid, **ID**, internal diameter; **KN**, kinetin; **m**, metacentric; **M_1**, mutant generation-1; **M_2**, mutant generation-2; **MI**, metaphase-1; **MS**, mass spectrophotometry; **MS**, Murashige and Skoog; **N_6**, Chu; **NAA**, 1-naphthaleneacetic acid; **NC**, naturopathic care; **NCBI**, National Centre for Biotechnology Information; **NK**, Natural killer; **NMR**, nuclear magnetic resonance; **NN**, Nitsch and Nitsch; **NPGS**, nanometer pattern generation system; **NPK**, nitrogen phosphorous potassium; **PCA**, principal component analysis; **PCR**, polymerase chain reaction; **PCV**, phenotypic coefficient of variation; **PGR**, plant growth regulator; **PI**, psychotherapy intervention; **PMC**, pollen mother cell; **RAPD**, randomly amplified polymorphic DNA; **RP-HPLC**, reverse phase-high performance liquid chromatography; **SAMPL**, selectively amplified microsatellite; **sc**, Secondary constriction; **SIM**, selected ion mode; **sm**, sub-metacentric; **st**, sub-telocentric; **TDZ**, thiadiazurone; **TF**, total frequency; **TLC**, thin layer chromatography; **UPGMA**, unweighted pair group method of arithmetic averages; **UV**, ultra violet

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INTRODUCTION

Withania somnifera (L.) Dunal (Family: Solanaceae), commonly known as Ashwagandha (English name: winter cherry) is a perennial (Ali *et al.* 1997) plant species (herb: Gupta *et al.* 1996; Khanna *et al.* 2006; Gupta and Rana 2007; undershrub: Flora of Hassan District Karnataka, India; shrub: Patra *et al.* 2004; Negi *et al.* 2006) with immense therapeutic uses. The medicinal properties of *W. somnifera* are due to its chemical constituents (alkaloids and withanolides) present primarily in roots, and also in leaves. The species is also known as 'Indian ginseng' as its roots possess restorative properties similar to *Panax ginseng*. An overview on *W. somnifera* covering nearly all essential aspects are presented in the text with the objective of providing an unabridged repository of references to pre-

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Review

sent and future researchers looking to eugenize the species for proper utilization in human welfare.

COMMON NAMES AND SYNONYMS

Ashwagandha (source: Herbs, Commerce 2nd Edn)- Sanskrit name meaning 'smell like a horse'; Winter Cherry (source: F Zamb)- English (no accession in NPGS are available). Coqueret somnifera, poc-poc sauvage, ti-poc-poc (French); Erva moura somnifera, pontadeira, malagueta de galinha, uva caneca (Portugese); Mtemua shamba, mhulapori (Swahili) (PROTA 2008). Synomymns: *Physalis somnifera* L. (basionym); *Withania sicula* Lajac. (Germplasm Resource Information Network 2010).

DISTRIBUTION

The species is widely distributed (ARS Systematic Botanists 1999) in Africa (extending from the Canary Islands to Cape Province including also East and West Tropical Africa) and Asia (temperate- Arabian Peninsula, Western Asia; tropical- Indian subcontinent) apart from its occurrence in Europe (Southeastern Europe-Greece and Italy; Southwestern Europe- Spain) and Western Indian Ocean (Mauritius). In India the species is reported from subtropical and semi-temperate regions but seldom occur in North Eastern parts (Singh and Kumar 1998).

THERAPEUTIC USES

W. somnifera is an ancient plant with immense therapeutic uses in traditional (Ayurveda, Sidhdha and Unani) and modern system of medicine.

Traditional uses

The root and root powder and paste of the species are important in the treatment of rheumatic pain, arthritis and cardiopulmonary disorder (Asthana and Raina 1989); constipation and loss of memory (Watt 1972); pulmonary tuberculosis (Maithani 1973); abortion (Sahu 1982); inflammation of joints, nervous disorder and epilepsy (Tiwari *et al.* 1979; Singh and Kumar 1998); glandular swelling, bubonic plague (Bhandari 1992), various physiological disorders (Singh *et al.* 1982); ulcers and tumours (Kapoor 2001); heart diseases (Malhotra *et al.* 1981; Bandyopadhyay and Jha 2003; Mohanty *et al.* 2004) amongst others. The drug is also suggested to be an aphrodisiac, diuretic, restorative and rejuvenative in nature (Tripathi *et al.* 1996; Patra *et al.* 2004; Kumar *et al.* 2007). The crushed leaves of *W. somnifera* are applied in external pains and inflammation (Sudhir *et al.* 1986; Dafni and Yaniv 1994), painfull joints and boils (Sharma *et al.* 1992) and also used for pubonic pains (Apparananthan and Chelladurai 1986).

Application in modern medicine

The species possess antioxidant (Bhattacharya *et al.* 2001; Gajera *et al.* 2005; Gupta and Rana 2007; Udaya Kumar *et al.* 2010), anti-depressant (Gupta and Rana 2007), anti ulcerogenic (Bhatnagar *et al.* 2005), antivenom (Lizano *et al.* 2003; Machiah *et al.* 2006), anti-inflamatory (Somasundaram *et al.* 1983; Anbalagan and Sadique 1984; Begum and Sadique 1987; Al-Hindawi *et al.* 1992), anti tumour (Singh *et al.* 1986; Prakash *et al.* 2002, Jayaprakasam *et al.* 2003; Ichikawa *et al.* 2006), antimasculine (Ilayperuma *et al.* 2002; Arora *et al.* 2004; Owais *et al.* 2005), antiparasitic (El-On *et al.* 2009), antimicrobial (Girish *et al.* 2006; Motara 2007; Kambizi and Afolayan 2008), anticancerous (Kataria *et al.* 2009; Maitra *et al.* 2009; Koduru *et al.* 2010; Muralikrishnan *et al.* 2010; Yu *et al.* 2010) and antiaging (Widodo *et al.* 2009) properties. The drug is effectively used to control Parkinson's (Naidu *et al.* 2003; Ahmad *et al.* 2005; Kumar and Kulkarni 2006; Raja Sankar *et al.* 2009) and Alzeimer's (Jayaprakasam *et al.* 2009) diseases apart from giving stress relief (Kour *et al.* 2009; Das *et al.* 2010), controlling insomnia (Bhattacharya *et al.* 2003) and improving memory by elevating acetyl choline (Schliebs *et al.* 1999). The species is also noted to possess neuro regenerative (Zhao *et al.* 2002; Tohda *et al.* 2005; Kumar and Kumar 2009) and immunomodulatory (Davis and Kutton 2000; Iuvone *et al.* 2003; Khan *et al.* 2009) properties.

In recent years, the plant species is widely applied in clinical trials for human beings. Ahmad *et al.* (2010) investigated the impact of *W. somnifera* roots on semen profile, oxidative biomarkers and reproductive hormone levels of infertile men (75 healthy fertile men as control and 75 infertile men-were patients; HPLC procedure was used for quantification of vitamin A and E in seminal plasma while the biochemical parameters in seminal plasma were estimated by standard spectrophotometric procedure). Results indicated that *W. somnifera* inhibited lipid peroxidation and protein carbonyl content and improved sperm count and motility. The authors concluded that the herbal preparation effectively reduced oxidative stress and improved semen quality. Cooley *et al.* (2009) explored the effectiveness of neuropathic care (NC) on anxiety symptoms by a randomized trial (NC-41 individuals; psychotherapy intervention-PT, 40 individuals) over a period of 12 weeks using herbal medicine, Ashwagandha (300 mg b.i.d. standardized to 1.5 withanolides prepared from roots). Result indicated significant differences between groups in relation to mental health, concentration, fatigue, social functioning, vitality and overall quality of life with NC group exhibiting greater clinical benefit. No serious adverse reactions were observed in either groups. Mahdi *et al.* (2009) studied the effect of root powder of *W. somnifera* (5 g/d for 3 months) on different groups (normozoospermic heavy smokers-20; normozoospermic with infertility of unknown etiology-20; normozoospermic fertile men as control-60) of individuals using biochemical and stress parameters before and after treatments. Treatments resulted in a decrease in stress, improved the level of anti-oxidants and improved overall semen quality in a significant number of individuals. Semen quality improved pregnancy (14%) in the partners. Mikolai *et al.* (2009) studied the effect of Ashwagandha root extract (6 ml of root extract was administered twice daily for 96 h with whole milk, anupana) on 4 types of immune cells in a human sample to determine the immunological mechanism (peripheral blood samples were collected at 0, 24 and 96 h and compared for differences in cell surface expression of CD4, CD8, CD19, CD56 and CD9 receptors by flow cytometry). Results indicated significant increase in the expression of CD4 on CD3+ T cells after 96 h. CD 56+ NK cells were also activated after 96 h as evidenced by expression of the CD69 receptor.

PLANT DESCRIPTION

The plant description (**Fig. 1**) is based on its cultivation in experimental field plots of the University of Kalyani, West Bengal plains (22° 99′ N, 88° 45′ E, elevation – 48 feet above mean sea level, sandy loamy soil, organic carbon 0.76%, soil pH 6.85) during the period of September to February (max. temp. 24.02-32.17°C, min. temp. 11.24-25.51°C; rainfall 0.0-12.46 mm; relative humidity max. 93.96-98.28%, min. 44.43-86.85%). The species is perennial (can be sown twice a year, in March to August and September to February; Das *et al.* 2009a), branched, terrestrial herb (plant height: 69.58-75.22 cm); tap root (**Fig. 2**), long (17.52 to 19.28 cm), unbranched, 2-3 cm thick, terate, colour (pale straw 12161; British Atlas of Colour, 7th edition, 2007), rootlets many, confined within 1-5 cm, angular and branched; stem terete, hairy to the younger part and glabrous at maturity; leaves alternate, simple, ovate to broadly ovate (7.0-12.0 cm long; 5.0-7.0 cm broad), acute at apex, entire, broad as well as cuneate at base, herbaceous, unicostate with 4-5 pinnate secondaries, hairy on both surface, green (colour code: 12163); petioles slender 1-2 cm long, hairy; flowers axillary in fasicles of 2-5 often 2-3 developed,

complete, bisexual, hypogynous, actinomorphic, pentamerous, pale green (24634-1), pedicillate; pedicel 2 to 3 mm long hairy; ebractiate; calyx cupipular to campanulate; sepals 5 lobes, triangular acute, about 1 mm long, tubular part about 2 mm long, hairy throughout, persistent and accrescent at maturity covering the whole fruit increased up to 1.5-1.8 cm long; corolla tubular, campanulate; petals 5, ovate triangular about 1.5 mm long, hairy on both surface, tubular part about 2 mm long; stamens 5, filaments slender, epipetalous and alternipetalous attached below the middle of the corolla tube, white glabrous; anthers oblong, two celled dorsifixed, dehiscence longitudinal-latrorse, white (11119); pollen grains (**Fig. 3**) prolate spheroidal (as per Erdtman 1952), fertility 59.96-71.05%, size 21.67 µm × 18.67 µm (18.5 pollen grains per microscopic field: 85564.29 μm^2); carpels 2 syncarpous; ovary oblong about 1.2 mm long, greenish, glabrous; style one terminal, terete about 1-5 mm long, glabrous; stigma rounded, ochre (16283); ovary two chambered with many ovules (size: 36.15 µm × 22.7 µm) per chamber (**Fig. 4**), in axile placentation; fruits berry, subglobose to rounded, coloured ('Poshita': fruit colour-chrome 11309 and 'Jawahar 22'-fruit colour-orange 11261) at maturity, many seeded, 4 to 5 mm across, glabrous with varied seed colour, fully covered with accrescent calyx; seeds flat (size: 2.2 mm × 1.6 mm), compressed and sub-reniform to coiled about 1.0 mm across, surface reticulate, glabrous, light brown to yellowish brown ('Poshita': sunset yellow 14913 and 'Jawahar 22': sunset yellow 14916).

NOTABLE VARIETIES

'WS 20' (Nigam *et al.* 1991) is a selection variety which recorded significant increase of 29.9 and 28.3% in dry root yield over 'Manasa Local' and 'Kukedesher Local', respectively. The roots contain 0.27% total alkaloids and the variety showed average stability over a wide range of environment. The proposed name of 'WS 20' on release was 'Jawahar Ashgand 20'. 'Poshita' is also a selection variety with improved root yield (14.04 q (quintals)/ha) and better chemical quality (total alkaloids: 1.292 kg/ha; total withanolides: 3.496 kg/ha) and it is developed in CIMAP, Lucknow, India (Misra *et al.* 2001). 'Rakshita' is another variety released from CIMAP and the variety yields about 8-10 q/ha dry roots with alkaloid content of the roots at about 0.5% (Mathew *et al*. 2005).

INTRASPECIFIC VARIABILITY

Atal *et al.* (1978) reported 5 different forms (**Form I:** cultivated in black soil of Madhya Pradesh; plant height 30 cm; root unbranched or slightly branched, soft, tuberous, starchy, colour light pale brown; bark thin, pale brown; branching lax; leaf obtuse tapering, wavy, hairy with inconspicuous veins; flower 5-partite; fruit up to 15 at a node; **Form II:** wild growing in sandy dessert of Pillani, Marwar and other parts of Rajasthan; height 0.6 to 1.5 m; root unbranched, erect, soft, tuberous, colour light pale brown; bark smooth, pale brown; branching lax; leaf sharply acute, entire, veins faint; flower 5- partite; fruit 1-2 at a node; **Form III:** plants growing in Chandigarh and Uttar Pradesh; plant height 0.6 to 1.0 m; root unbranched, erect and texture and colour corresponds closely to form II; branching heavy; leaf sharply acute entire with dense hairs, veins faint; flower 5- fid; fruit 5-7 at a node; **Form IV:** plants growing near Delhi and Punjab; plant height 0.6 -0.75 m; root with prominent lateral branches, hard, woody, reddish brown; bark course, thick, brick red; fruit 5-10 at a node; and **Form V:** wild growing near Delhi and Ahmedabad; plant height 1.2-2.1 m; root more or less similar to form IV; branching lax, leaf acute, entire, veins faint, hairs moderately present; flower 5- partite; fruits up to 15 at a node) of *W. somnifera* on the basis of its distributional range and salient morphological features in India (23° N to 33° N; 180 m to 1700 m above sea level). Roots of form I was considered to be of commercial importance (Datta and Mukerji 1950; Loges and Neuwald 1956; Atal *et al.* 1975).

REPRODUCTIVE BIOLOGY

Kaul *et al.* (2005) studied the morphological and functional characteristic of flowers contributing to the reproductive success in AGB-002 (wild morphotype from Rajasthan, India) of *W. somnifera* and for which pollen transfer experiment were conducted to explore the effects of pollen source on fruit set and seed germination following autogamy, open pollination and xenogamy. Results indicated that autogamy and open pollination resulted in high fruit set (81.83 and 86.66%) compared to xenogamy (3.59% on average). Synchronous stigma receptivity, anther dehiscence and close proximity of both male and female organs predisposed the species to autogamy. Pollination occurs on the day of anthesis and fertilization on the next day. High pollen (viability: 90.5% at peak flowering season-April-June, 80 to 83%, during post monsoon-September; pollen grains remained viable for 24 hour under field conditions) load (approx. 817) on stigma reduces the possibility of cross pollination. On the contrary, Lattoo *et al.* (2007) were of opinion that floral (high pollen-ovule ratio 631.77; high seed-to-ovule ratio: 0.76; moderate fruit-to-flower-0.49) phenoevents suggested that crossing and autonomous selfing are mutually exclusive as the self-pollen arrives late during floral ontogeny and consequently seed-set efficiency and fruiting success were not influenced by pollen genotypes (self/cross) under different pollination treatments (autogamy, geitonogamy, and xenogamy). The authors further suggested that mixed mating and efficient recombination system provide the reliability of reproductive success and genetic polymorphism.

Mohan Ram and Kamini (1964) suggested the formation of a globular embryo in *W. somnifera*, which is *Solanad* type. The embryo sac development is a *Polygonum* type.

ANATOMY

Stem

Transverse section (4.0-5.0 cm above base) of stem (main stem, 75-days-old plant at vegetative stage; hand sectioned and double stained as per Johansen 1940) showed the following features (**Fig. 5**): epidermis 1 cell layer thick, cells more or less rounded to barrel shaped rarely rectangular, medianly thick walled, compactly arranged; hypodermis 1 cell layered thick, cells compact, rounded, medianly thick, smaller than the epidermal cells, at maturity hypodermal layer may be indistinct; cortex 6-8 cell layered thick, cells rounded, smaller towards periphery, gradually larger and again smaller towards stele, thin walled loosely arranged with intercellular spaces; stele consisting of a ring of vascular structure having pith to the centre, siphonostelic; outer phloem layer few cell layered thick, continuous as well as in patches; cambium 2-3 cell layers thick, inconspicuous; xylem layer larger with medullary rays; inner phloem in discrete patches; pith cells alike to cortical cells.

Root

Transverse section (main root of 75-days-old plant, 5-6 cm below the top; double stained) of root (**Fig. 6**) documented the following: periderm (phellem, phelogen and phelloderm: 3 layers evident) present; phellem 3-5 cell layered thick, tetra to polygonal, thin walled, compactly arranged with contents within; phellogen 2-3 cell layered thick, rectangular, compactly arranged; phelloderm 5-7 cell layered, rectangular thin wall, compactly arranged; cortex 7-10 layered thick, polygonal to rounded often with brownish content within; pericycle and endodermis indistinct due to secondary growth; stele prominent with distinct xylem and ray cells; primary vascular bundle tetra-arched, centrally placed with metaxylem and phloem arranged radially.

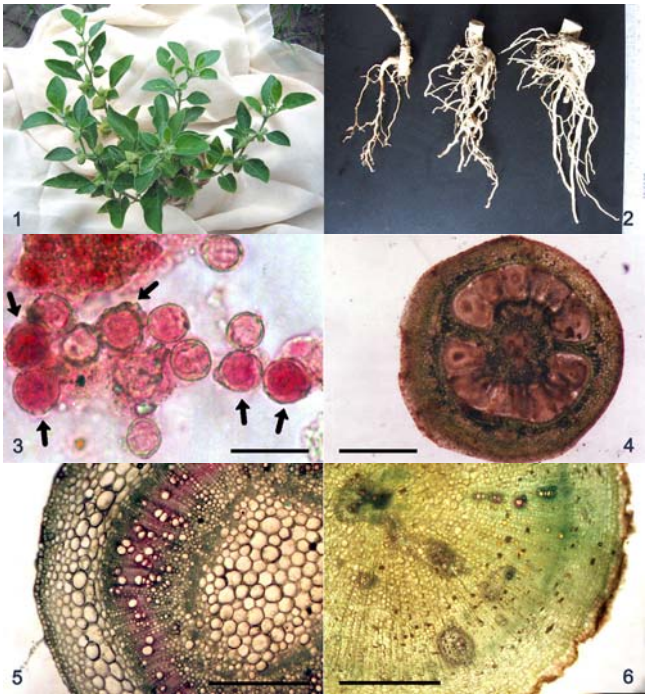

Figure plate I (**1-6**) *W. somnifera.* (**1**) The whole plant of *W. somnifera*; (**2**) Roots with rootlets, (**3**) Fertile (fully stained →) and sterile pollen grains (scale bar = 100 µm); (**4**) T.S. of ovary showing two chambers with many ovules (scale bar = 1 mm); (**5**) T.S. of stem (scale bar = 5 mm); (**6**) T.S. of root showing secondary activity. Primary vascular bundles evident (scale bar = 5 mm).

CULTIVATION

In natural conditions *W. somnifera* occurs on disturbed soil, along roadsides, in cultivated land, on termite mounds in grassland, in open woodland and riverine vegetation, from sea level upto 2300 m altitude. It is grown in areas with 600 – 750 mm annual rainfall and prefers well drained soil; waterlogging is harmful (Patra *et al*. 2004). It grows well in sandy loams and stony red clay soils with pH 7.5–8.0 (Thomas *et al*. 2000). However, Obidoska and Sadowska (2003) suggested the preference of the species to acidic soil. It thrives in full sun but tolerates some shade (PROTA 2008).

Patra *et al.* (2004) suggested that the species is a rainfed crop requiring dry season during its growth period (1-2 winter rains are conducive for root development) and grows well in semi-arid subtropical areas receiving good rainfall. The species can be cultivated between 600-1200 m altitudes. Misra *et al.* (2001) suggested that *W. somnifera* is a potential cash crop greening dry-land zones and making wasteland more productive. Das *et al.* (2009a) successfully cultivated 'Poshita' and 'Jawahar 22' (recommended varieties) in West Bengal plains (Bidhan Chandra Krishi Viswavidyalaya, Mohanpur campus, Nadia, 22° 56′ N latitude, 88° 32′ E longitude and 9.75 m altitude; sandy loamy soil, organic carbon-0.76%, soil pH 6.85) in two seasons (March to August as rainfed kharif crop; September to February as rabi crop).

Land preparation

Traditional land preparation consists of several ploughings and planking and subsequent levelling to prepare weed-free seed bed. In nursery seeds are sown at the onset of rains at the rate of 2-5 kg/ha (PROTA 2008) seed treatment with a fungicide (Bavistin^{TM} – 0.1%) is recommended before sowing (Das 2010).

Plantation

The species is propagated by seeds either by broadcasting method (10-12 kg/ha) or by line sowing keeping uniform distance between plants (5-10 cm) and rows (20-25 cm). Line sowing method increases the root production and also helps in performance of intercultural practices (Patra *et al*. 2004). Plant density depends on the nature and fertility of soil. Seeds are also germinated in nursery beds and subsequently six weeks-old seedlings are transferred to field plots and sown in lines keeping 60 cm apart (Iqbal 2007).

Germination

Hussain and Ilahi (1988) found that the freshly collected seeds of *W. somnifera* did not germinate well probably due to immaturity of embryo in them and therefore requiring a post harvest maturation period. The authors further suggested that 8-12-months-old seeds exhibited 70-80% germination with application of indole-3-acetic acid (IAA), gibberellic acid (GA_3) or thiourea alone or in combinations with mechanical scarification at 25°C. Similarly, soaking in a nutrient solution or water alone and subsequent to scarification enhanced germination by 50%. Kattimani *et al*. (1999) suggested that presoaking of Ashwagandha seeds in 1% sodium nitrate gave the most rapid germination and highest percentage of germination (92% compared with 26% in untreated control). Verma *et al.* (2000) suggested that seeds of *W. somnifera* treated with 100 ppm GA_3 resulted in improved percentage of germination, speed of germination, emergence and coefficient of velocity of germination, and a reduced mean germination time, compared with control seeds. Suryawanshi *et al.* (2001) reported that seeds of *W. somnifera* germinated better in between papers and the highest germination frequency of 92% was obtained at 20°C. Mitra and Ghosh (2004) reported that seeds treated with 50 ppm GA_3 gave the highest germination percentage (83.33%), speed of germination, coefficient of the velocity of germination (11.55%) and the lowest mean germination time (8.66 days). Kambizi *et al.* (2006) suggested that germination in the species was temperature and light dependent.

Fertilizer response

Application of organic manures like, Farm yard manure (FYM), vermi-compost, and green manure amongst others are found beneficial for maximizing yield (Patra *et al.* 2004). Moderate application of nitrates (50 kg/ha) and phosphates (25 kg/ha) increased root yields more than 800 kg/ha and the quality of root yields were thicker (PROTA 2008). Maitra *et al.* (1998) reported that application of NPK (28: 28: 28 g/1.3 m^2) gave the best (and significant) increase in vegetative growth (root yield specifically), flowering and fruiting. Muthumanickam and Balkrishnamurthy (1999a) suggested that treatment with 40 kg N, 60 kg P_2O_5 and 20 kg K_2O/ha gave the highest dry root yield of 770.3 kg/ha and highest withanolides content of 0.49%. Maheshwari *et al.* (2000) noted the response of Ashwagandha to organic manures and fertilizers in shallow black soil under rain-fed condition and suggested that the highest dry root yield, net returns and cost: benefit ratio were obtained by applying 2.5 tonnes/ha FYM + 12.5 kg nitrate/ha + 25 kg phosphate/ha.

Irrigation

If required irrigations may be applied, but excessive water in the form of rainfall or irrigation is considered detrimental for growth of the species (Singh and Kumar 1998; Patra *et al*. 2004; PROTA 2008; Das 2010).

Yield

Muthumanickam and Balkrishnamurthy (1999b) reported that the optimum stage for extraction of total withanolides content in Ashwagandha is 6-month-old plants (0.61%) and among different organs, roots had the highest withanolides (0.30%) followed by leaves (0.15%) and stems (0.08%).

Kattimani and Reddy (2001) found that soaking seeds in 1% sodium nitrate resulted in longest root (15.99 cm) followed by 0.5% zinc nitrate (13.95 cm) and 1% potassium nitrate (13.73 cm). Seeds soaked in 1% sodium nitrate recorded the highest dry root yield (131.03 kg/ha), followed by those soaked in 1% potassium nitrate (114.35 kg/ha) and 0.5 % calcium nitrate (110.02 kg/ha). Barathkumar *et al.* (2001) were of opinion that growth and yield attributes were favourably influenced by the application of phosphobacteria along with plant growth regulators except maleic hydrazide at the rate of 5000 ppm. Kothari *et al.* (2003) showed that morphology, yield and quality of Ashwagandha roots are influenced by tillage depth and plant population density. Agarwal *et al.* (2003) suggested that sowing time and spacing are important criteria for economic evaluation of the species. Similarly, Desai *et al.* (2004) were of opinion that seed yield and quality of Ashwagandha were influenced by sowing dates. Patel *et al.* (2003) showed that harvesting 210 days after sowing resulted in maximum root yield (277 kg/ha). Pol *et al.* (2003) indicated that growth and yield of *W. somnifera* advanced with foliar spray of growth regulators and micronutrients in variable concentrations. In India yield of *W. somnifera* is about 650-800 kg/ha of fresh root (150-350 kg/ha dry roots) and on an average of 300-500 kg/ha (PROTA 2008).

Storage and shelf life

Patil *et al.* (2010) studied physiochemical stability and biological activity of *W. somnifera* dried root aqueous extract during six months real-time and under accelerated storage condition. HPLC-DAD method for quantification suggested a significant decline in withaferin A and withanolide A content under real and accelerated conditions. Change in chemical content was concurrent with a significant decline in immunomodulatory activity ($p<0.01$) during third month of the accelerated storage. The authors suggested that adequate control of temperature and humidity are essential during storage and shelf life of the species.

DISEASES AND PESTS

Ashwagandha is reported to be affected by leaf spot (*Myrothecium roridium* and *Pseudocercospora withanii*; Aneja and Kaur 1995; Mahrshi 1986; *Alternaria alternata*; Pati *et al.* 2008), die-back (*Alternaria alternata*; Gupta *et al.* 1993), root-rot and wilt (*Fusarium solani*; Gupta *et al.* 2004). Witches-broom (*Phytoplasmas*; symptoms: stunted growth, reduced leaf size, axillary bud proliferation and reduced inter-nodal length; control: tetracycline hydrochloride spray gives partial recovery but uprooting and burning of the infected plants were recommended to avoid spread) and tobacco leaf curl (leaf shows vein clearing and rolling and low seed set; removal of infected plants avoid spread) diseases were also reported in the species (Patra *et al.* 2004; Samad *et al.* 2006). Seedling blight, leaf blight, damping off and seed rotting were also noted in seedling stage (control: seed treatment with Thiram™ or Deltan™ at 3-4 g/kg seed coupled with 1 or 2 sprays of 0.3% Fytolon®, Dithane Z-78 or M-45) of Ashwagandha (Patra *et al.* 2004).

Lady beetle (*Epilachna vigirtioctopunctata*) causes severe attack on leaves (pale-coloured and subsequently dried up; control measures: foliar spray 10-15 days interval of 0.5% Malathion and 0.1-0.3% Kelthane) and nematodes on roots (Carofuran at the rate of 5-6 kg/ha recommended at the time of sowing) were reported in *W. somnifera* (Patra *et al.* 2004).

BIOCHEMICAL ANALYSIS

Primary metabolites

Khanna *et al.* (2006) studied soluble protein, total amino acid, reducing and non-reducing sugar and starch contents in fresh roots of five selective accessions (AGB-002, 009, 015, 025, 030) of *W. somnifera*; while, crude fiber content was also analyzed in dry roots during young and maturity stages. Results indicated that all the accessions followed a uniform pattern of maximum biochemical constituent accumulation during maturity, and AGB 002 responded best. Further, the authors were of the opinion that root with less fibre content were mostly preferred and exploited for commercial purpose. High fibre content noted in the root was attributed to delayed harvesting of the plants. All the biochemical parameters showed positive correlation with root yield and therefore were of commercial interest.

Secondary metabolites

1. Alkaloids

Power and Salway (1911) isolated an amorphous alkaloid ($C_{12}H_{16}N_2$) from root of *W. somnifera* with no marked physiological activity. From Bengal source the drug alkaloid was confirmed from the species (Majumdar and Guha 1933). Majumdar (1955) established the presence of 8 brown alkaloids (nicotine, somniferine, somniferinine, somine, with anine, pseudo-withanine, withananine, withana-ninine), the major alkaloid being withanine which showed marked sedative and hypnotic action. The chromatographic analysis of root extracts confirmed the presence of a number of biochemically heterogenous alkaloids. Through chromatography, 13 Dragendorff-positive components in the root (Covello and Ciamapa 1960; Rother *et al.* 1961; Khafagy *et al.* 1962; Khanna 1963) and 5 in leaf (Dhalla *et al.* 1961) were obtained. Dragendroff-positive alkaloids reported were anaferine [bis (2-piperidylmethyl) ketone], isopelletierine, tropine, pseudotropine, 3-tigloyloxtropine, 3-tropyltigloate, cuscohygrine, dlisopelletierine, anahygrine, hygrine, mesoanaferine, choline, somniferine, withanine, withananine, hentriacontane, visamine and withasomnine (Morimoto *et al.* 1968; Trutneva 1971; Gupta and Rana 2007). The total alkaloid content of Indian roots of *W. somnifera* was reported to vary between 0.13 to 0.31% but sometimes yield up to 4.3% (Singh and Kumar 1998). The wide variation in the yield of alkaloid may be due to several factors, such as method of isolation used, genotype variability in the species and genotype/environmental interactions (Srivastava *et al*. 1960; Schwarting *et al*. 1963). Individual alkaloids were not found to possess any specific biological activity; only the total alkaloids possess therapeutic uses (Singh and Kumar 1998).

2. Withanolides

Power and Salway (1911) revealed the presence of a compound withanol ($C_{25}H_{34}O_5$) from roots and somnirol ($C_{32}H_{44}O_5$) and somnitol ($C_{33}H_{46}O_7$) from leaves of *W. somnifera*. Kurup (1958) isolated the first unsaturated lactone ($C_{24}H_{30}O_6$) from the leaves of Ashwgandha and was confirmed to be withaferin A (Lavie *et al.* 1965; Yarden and Lavie 1962). The leaves (mainly) and roots of the plant species contain different withanolides – a group of C_{28} steroid characterized by 6-membered lactone ring in 9 carbon atom side chain, and differing in substitution pattern (Abraham *et al.* 1975; Eastwood *et al.* 1980; Velde and Lavie 1981). This group of compounds includes ergostane-type steroidallactones namely withaferin A, withanolides A-γ, withasomniferin-A, withasomidienone, withasomniferols A-C, withanone amongst others. The withanolides isolated and identified are been documented in **Table 1**.

Spectral analysis identified new withanolides namely 5-dehydroxywithanolide-R and withasomniferin-A from aerial parts (Rahman *et al.* 1991), withasomidienone from the whole plant (Rahman *et al.* 1992) and 5β, 6α, 14α, 17β, 20β–pentahydroxy-1-oxo-20S, 22R-witha-2, 24 dienolide and 6α, 7α–epoxy-5α 14α, 17α, 23β–tetrahydroxy-1-oxo-22R-witha-2, 24 dienolide from fruits (Abou-Douh 2002) of *W. somnifera.* Choudhrary *et al.* (2004) through spectral analysis identified two new withanolides of *W. somnifera*

Table 1 Chemical constituents of *W. somnifera.*

Name of the compound	References
Dihydrowithaferin A	Lavie *et al.* 1965
27-deoxy-14α hydroxy withaferin A	Glotter *et al.* 1966
Withanolide D	Lavie *et al.* 1968
a) 27-deoxywithaferin A	Kirson *et al.* 1971
b) 4β-hydroxy-5β, 6β-epoxy-1-oxo-22R-witha-2,14-24-trienolide	
Withanolide F, I, J, K, L, M	Glotter *et al.* 1973
a) 27-deoxy-17α hydroxy withaferin A	Abraham *et al.* 1975
b) Withanolide N and O	
Withanolide G	Kundu *et al.* 1976
Withanolide P	Glotter *et al.* 1977
a) 4β, 20α-dihydroxy-1-oxo-5β, 6β, epoxy-20R, 22R-witha-24 enolide	Eastwood *et al.* 1980
b) 20α-hydroxy-1,4-dioxo-5β, 6β, epoxy-20R, 22R-witha-2, 24 dienolide	
c) 20α-hydroxy-1,4-dioxo-5β, 6β, epoxy-20R, 22R-witha-2 enolide	
a) 1α,3β,20 α_F- trihydroxy-20R; 22-R-witha-5-24-dienolide	Velde and Lavie 1981
b) 1-oxo-14α, 20α_F, 20R, 22R-witha-3, 5, 24-trienolide	
a) 5α-ethoxy-1-oxo-6β, 14α, 17β, 20-tetrahydroxy-20S, 20-R-witha-2, 24-dienolide	
Withanolide E and H	Ozguven *et al.* 1991
Withanolide C	Bessalle and Lavie 1992
17-isowithanolide E	Vitali *et al.* 1996
3α-methooxy-2, 3-dihydro-27 deoxywithaferin A	Anjaneyulu and Satyanarayana Rao 1997

namely 6α, 7α-epoxy-3β, 5α, 20β-trihydroxy-1-oxowitha-24-enolide and 5β, 7β-epoxy-4β, 17α, 27-trihydroxy-1-oxo-witha-2, 24-dienolide and demonstrated cholinesterase activity of said withanolides.

3. Quantification methods of secondary metabolites

Gupta *et al*. (1996) quantitatively determined Withaferin A in different plant parts of *W. somnifera* by TLC densitometry and reported its presence only from leaf samples. Ganzera *et al*. (2002) quantified withaferin A and withanolide D from different plant parts of the species following HPLC analysis while Mahadevan *et al*. (2003) presented a reproducible, accurate and precise HPTLC method for estimation of withaferin A by using precoated silica gel G (aluminium backed) plates as stationary phase and toluene: ethyl acetate: formic acid (50: 15: 5) as mobile phase. Detection and quantification of the compound were made from densitometric readings at λ_{max} 213 nm with linearity range of 1 to 3 µg. Khajuria *et al.* (2004) also described a method for separation, identification, and quantification of withanolides from plant extracts using HPLC-UV (DAD)-mass spectrophotometry (HPLC-Mass) with Merck (250 × 4.6 mm ID, 5 µm) column and analyzed by electrospray ionization on a mass spectrophotometer in selected ion mode (SIM). For quantification $[M^+Na]+$ ions were monitored and linear calibration curves were obtained in concentration range of 1.50 to 6.5 µg/ml. A simple technique was suggested by Dalavayi *et al*. (2006) for determination of withaferin A with RP-HPLC using acetonitrile water as mobile phase. Srivastava *et al.* (2008) described a sensitive, selective and robust desitometric HPTLC method for simultaneous determination of three key withanolides (withferin A, 12-deoxytranmonoline and withanolide A) in Ashwagandha plant samples. The separation was performed on aluminium backed silica gel 60F254 HPTLC plates using dichloromethane-methanol-acetone-diethyl ether (15: 1: 1: 1) as mobile phase at λ_{max} 230 nm. Nayak *et al.* (2009) also used HPTLC method for determination of withaferin A using toluene-ethyl acetate-formic acid – 5: 5: 1 as mobile phase and the adsorption was measured at 200 nm in reflectance mode.

CYTOGENETICAL STUDIES

Karyotype analysis

The chromosome number in *W. somnifera* is reported to be variable as 2n = 24 (Mohan Ram and Kamini 1964), 2n = 48 (Bhaduri 1933; Gottschalk 1954; Baquar 1967; Bir and Sidhu 1980; Iqbal and Datta 2007a; Das *et al.* 2009b) and 2n = 72 (Bir and Neelam 1980). Iqbal and Datta (2007a) revealed 7 ($2n=48:4A_{sm}^{sc}+4B_m+14C_m+4D_{sm}+2E_{st}+18F_m+2G_{sm}$) morphologically distinct chromosome types in the species (karyotype performed through image analyzing system) with prevalence of chromosomes with median primary constrictions (karyotype symmetric in nature- TF%: 42.26) and the chromosome length varies from 1.43 to 2.64 µm. Das *et al.* (2009b) performed karyotype analysis in 'Poshita' and 'Jawahar 22' (high performing recommended varieties) and documented 8 (chromosome length – 1.03 to 2.43 µm; satellites in two pairs; karyotype symmetric – TF%: 41.7) and 7 (chromosome length – 1.20 to 2.37 µm; satellites in two pairs; karyotype symmetric – TF%: 41.5) chromosome types respectively with preponderance of median chromosomes. Iqbal and Datta (2007a) and Das *et al.* (2009b) reported polysomatomy (2n = 6, 12, 18, 24, 36, 48, 72) in the species from temporary root tip squash preparations with predominance of 2n = 48 (**Fig. 7**). On the contrary Latto *et al.* (2007) reported that chromatin length in *W. somnifera* is 47.51 µm and is distributed asymmetrically among 24 chromosomes (2n = 48). Attributes of 2n and 4n *W. somnifera* plants are listed in **Table 2**.

Meiotic studies and basic chromosome number

Ray and Jha (2002) reported 21 pairs of bivalents in regenerated plants of *W. somnifera*. Iqbal and Datta (2007a) confirmed 2n = 48 chromosomes in the species from meiotic analysis (**Figs. 8, 9**) with an average of 23.52 II + 0.95I per cell at metaphase I. Das *et al.* (2009b) also suggested the presence of 2n = 48 chromosomes in meiocytes of the species ('Poshita': 23.67II + 0.67I/cell at MI; 'Jawahar 22': in and 23.69II + 0.62I/cell at MI) with prevalence of rod bivalents. Anaphase I (AI) distribution (**Fig. 10**) of chromosomes was nearly normal (24/24 separation) in 'Poshita' (87.91%) and 'Jawahar 22' (90.30%); while, the rest of the PMCs had laggards (9.27% to 9.89%) and bridge (0.46% to 2.20%) formation (**Figs. 11-13**). A persistent feature in metaphase I cells of *W. somnifera* was the presence of secondary association of chromosomes (bivalents and univalents tended to form variable groups of 3, 6, 9, 10 and 12) and statistical analysis of data suggested secondary polyploid nature of the species with x = 12 as its ancestral basic chromosome number (Iqbal and Datta 2007a; Das *et al.* 2009b). Lattoo *et al.* (2007) suggested high recombination index (71.2) in meiotic system of *W. somnifera* in accordance with the theoretical predictions for mixed mating thereby maintaining intermediate levels of heterozygosity. It was confirmed by RAPD analysis of polymorphic loci

Table 2 Attributes in 2n and 4n plants in *W. somnifera.*

Parameters	4n	2n
Plant height (cm)	40.0	50.4 ± 2.59
Leaf area (cm^2)	7.354 ± 0.438	5.694 ± 0.678
Chlorophyll content (mg/g of tissue)	chlorophyll *a*: 0.4273; chlorophyll *b*: 0.4998	chlorophyll *a*: 0.21365; chlorophyll *b*: 0.2499
No. of stomata in 85564.29 μm^2 microscopic field	9.2	16.08
Size of stomata (μm^2)	82.0 ± 2.88 × 63.5 ± 2.38	71.5 ± 2.26 × 58.5 ± 2.03
Days to flowering	105	90 to 100
Flower size (cm)	1.46 ± 0.073	1.1 ± 0.063
Fruit size (cm)	0.92 ± 0.0867	0.82 ± 0.0523
Mean number of ovules/ovary	8.2	11.4
Size of ovules (μm^2)	36.15 ± 0.65 × 22.7 ± 0.84	30.93 ± 1.47 × 21.21 ± 1.52
Seed per capsule	20.5	25.6 ± 1.564
Seed size (mm^2)	3.1524 ± 0.51 × 2.2089 ± 0.32	2.2 ± 0.46 × 1.6 ± 0.32
Chromosomal association	3 IV + 34 II +16 I 3IV+35II+14I	23.2 II +1.6 I per cell; 120 cells scored
No. of pollen in 85564.29 μm^2 microscopic field	10.2	18.5
Pollen size (μm^2)	24.67 × 21.67	21.67 × 18.67
Pollen fertility (%)	23.57	59.96-71.05
Withaferin A content (%)	1.46	1.23 (range 1.17-1.25)

based on Shannon index. Singhal and Kumar (2008) considered n = 12 and n = 24 as diploid and tetraploid cytotypes, respectively of *W. somnifera* from Punjab, Rajasthan and Himachal Pradesh has as a part of morphogenetic diversity in Indian population.

Pollen fertility analysis and morphology and ultrastucture

Pollen fertility assessed following staining (Marks 1954) of pollen grains in 1% propionocarmine was 59.96 to 71.05% (Iqbal and Datta 2007a; Das *et al.* 2009b). Alwadie (2002) studied the ultrastucture of *W. somnifera* pollen grains following the use of light, scanning and transmission electron microscopy. Light microscopic examinations revealed that the pollen grains were tri- or tetrazonocolpate, approximately as long as they are broad measuring 29 µm. Scanning electron microscopic observation showed two layers of pollen wall (exine-ektexine and endexine; intine) with surface sculpturing (scabrate-granulate), and containing numerous starch grain, lipid droplets, endoplasmic reticulum and vescicles of dictyosomes.

Spindle anomalies

Saeed *et al.* (1989) analyzed spindle anomalies in microsporogenesis in a population of *W. somnifera* from Karachi and found abnormalities including bridges, univalents and laggards. The authors noted multipolar and supernumerary spindles during second meiotic division resulting in 49 nucleate telophase II and subsequent pollen abortion.

Seasonal influence

Meiotic studies in *W. somnifera* during the months of October-November and May-June in West Bengal plain (University of Kalyani campus) demonstrated distinct variation between the flowering periods of various meiotic parameters (Mukherjee *et al.* 2006). Results indicated that the species was scheduled for its normal meiosis in October-November and shifting of its meiotic studies to May-June may not only find significant variation in the chromosome behviour at diplotene, MI and AI, but more importantly showed great variations in their meiotic products in terms of fertile pollen counts.

Mutagenic effect on meiotic chromosome behaviour

Iqbal and Datta (2005) analyzed effect of gamma-rays, and ethylmethane sulphonate (EMS) on chromosome behaviour of M_1 plants. Results suggested that average chromosome association at MI per cell (control: 23.52II+0.95I) varied from 21.78II+3.46I to 23.76II+0.48I in gamma irradiations and 23.13II+1.75I to 23.86II+0.28I in EMS). Univalents formed due to mutagenic treatments (mostly found to lie at close proximity to one another) bear no significant correlation with mean chiasmata/cell (r = -0.18 at 10 DF) thereby indicating that other bivalent might be compensating. Anaphase I and II were irregular in mutagenic treatments (formation of bridges, laggards, irregular separation of chromosomes and showed tendency of multipolarity) resulting to low pollen fertility (control – 70.3%, treatments – 24.9 to 68.5%).

Paracentric inversion

Iqbal and Datta (2006a) reported paracentric inversion was reported from the natural population of *W. somnifera* and such intrachromosomal gene rearrangement was attributed for widespread variability and polymorphism.

Cytomictic behaviour of chromosomes

Datta *et al.* (2005) reported persistent occurrence of cytomixis (chromatin/chromosome migration between and among meiocytes: **Fig. 14**) during microsporogenesis (recorded in prophase I and metaphase I cells; 2-4 cell clusters were formed) in control and mutagen (physical: X- and γ-rays; chemicals-EMS, dES and hydroxylamine at different doses)-treated M_1 plants resulting in either anucleated or with deficient and with extra chromosomes than normal. The nature of cytomixis was identical in the plant types assessed. The authors were of opinion that as the phenomenon of cytomixis was exhibited only by few groups of cells, it is logical to suggest that most of the meiocytes present in the microsporangium either vary in their cytomictic potential or fail to get exposed to the specific condition required to trigger the process. The authors further suggested that the individual meiocytes within the compact group being closely adpressed to facilitate, chromatin/chromosome migration. Singhal and Kumar (2008) reported cytomixis from diploid (n = 12) and tetraploid (n = 24) cytotypes of *W. somnifera* and the frequency was much less (15.80-24.32%) among tetraploids than diploids.

GENETICAL STUDIES

Studies on induced mutagenesis

Iqbal and Datta (2006b) studied mutagenic effectiveness and efficiency of gamma-rays, hydroxylamine (HA) and ethyl methane sulphonate (EMS) in *W. somnifera* based on M_1 biological damages (lethality, injury and sterility) and viable mutation frequency (M_2 generation) and suggested that HA and EMS were effective than gamma-rays; while

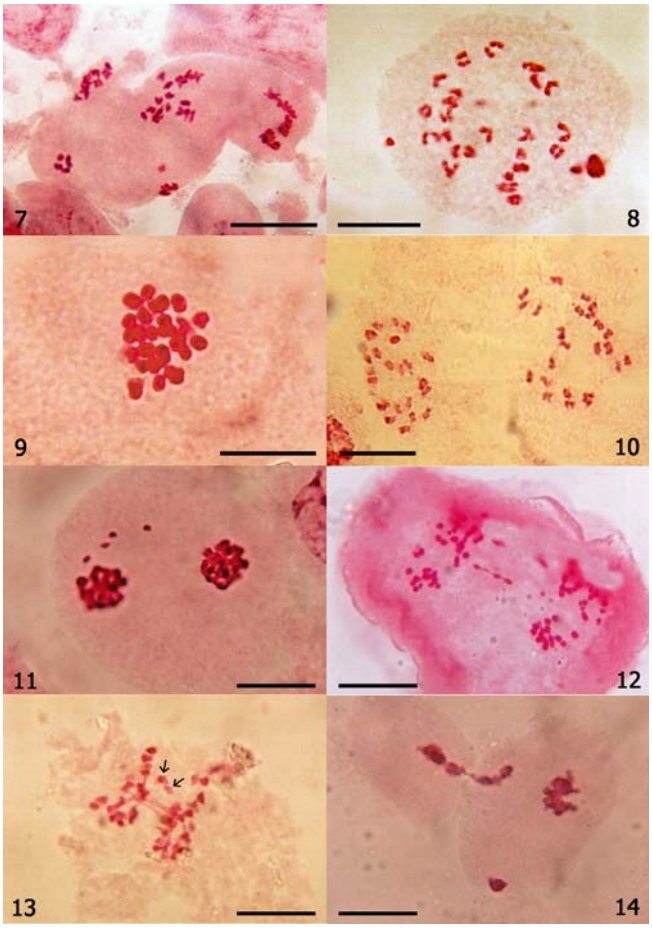

Figure plate II (**7-14**) showing cytological configurations of *W. somnifera*. (**7**) Metaphase cell with 2n = 48 chromosomes in groups; (**8**) 24II in diplotene; (**9**) 24II at MI; (**10**) 24/24 separation of chromosomes at AI; (**11-12**) AI with laggards; (**13**) AI showing bridge formation with associated fragments (→); (**14**) Chromatin migration between meiocytes (cytomixis) (scale bar = 10 µm).

lower doses of gamma-rays (2.5 and 5 kR) along with all the doses of chemical mutagens administered were found to be efficient. Iqbal and Datta (2007b) reported ten induced (different doses of gamma irradiations, HA and EMS) morphological mutants (*lax branching*, *bushy*, *dwarf*, *broad* and *ovate leaf*, *thick stem I* and *II*, *chloroxantha*, *early* and *late flowering*) at M_2. Normal and mutants had 2n = 48 chromosomes at metaphase I. Viable mutation frequency (high frequency estimated was due to low turn over of plants at M_2) was estimated to be highest in hydroxylarnine (21.18%) followed by gamma irradiation (18.86%) and EMS (17.65%). The mutant traits were found monogenic recessive to normal. Biochemical analysis (estimation of total alkaloid and withanolides and assay of catalase and antioxidant activities) revealed that the *broad leaf*, *bushy*, *thick stem I* and *II* and *ovate leaf* mutants were promising. Iqbal and Datta (2007c) also reported a selection line possessing *broad leaf* trait (2n = 48) with high root and seed yield. Das *et al.* (2010) reported eight macromutants in 'Poshita' and 'Jawahar 22' of *W. somnifera* following EMS treatments (0.25, 0.50 and 1.00% for 2 h and 4 h durations) to dry seeds. Viable mutation frequency was recorded to be 0.00-3.00% in 'Poshita' and 0.00-2.31% in 'Jawahar 22'. Analysis of morphological parameters (root and shoot attributes including seed yield), secondary metabolites (total alkaloid and withanolides; withaferin A and withanolide A by HPLC) and fibre content of the roots (withaferin A was also analyzed also from leaf) of M_3 plants revealed that 'bushy' and 'thick leaf' mutants were noteworthy mutants.

Polyploidy

The present authors (Das and Datta, unpublished) attempted to induce polyploidy (with an objective to widen the gene pool for improving chemical constituents specifically; perennial nature of the plant species was taken into consideration) in 'Poshita' and 'Jawahar 22' of *W. somnifera* by treating the meristematic tips of young seedlings bearing only two cotyledonary leaves with aqueous solution of colchicine (0.05, 0.1 and 0.25% treatments for 3 h on 1, 2 and 3 consecutive days; 5 seedlings were treated in each lot for each variety). None of the treated seedlings of 'Poshita' survived. One plant (0.02) of 'Jawahar 22' was cytologically confirmed (2n = 4x = 96) to be polyploid (**Fig. 15**) and the plant yielded seeds (straw - 12163). Different traits of 2n and 4n plants are listed in **Table 1**. A student's *t*-test was computed between the attributes at different degrees of freedom (DF) to assess the level of significance, if any. Chlorophyll content (as per Arnon 1949) in leaves (DF 8, $P < 0.001$), stomatal frequency (stomatal studies were made from leaf impression from lower surface as per Nayeem and Dalvi 1989) and sizes (DF 18, $P < 0.001$), fruit size (DF 8,

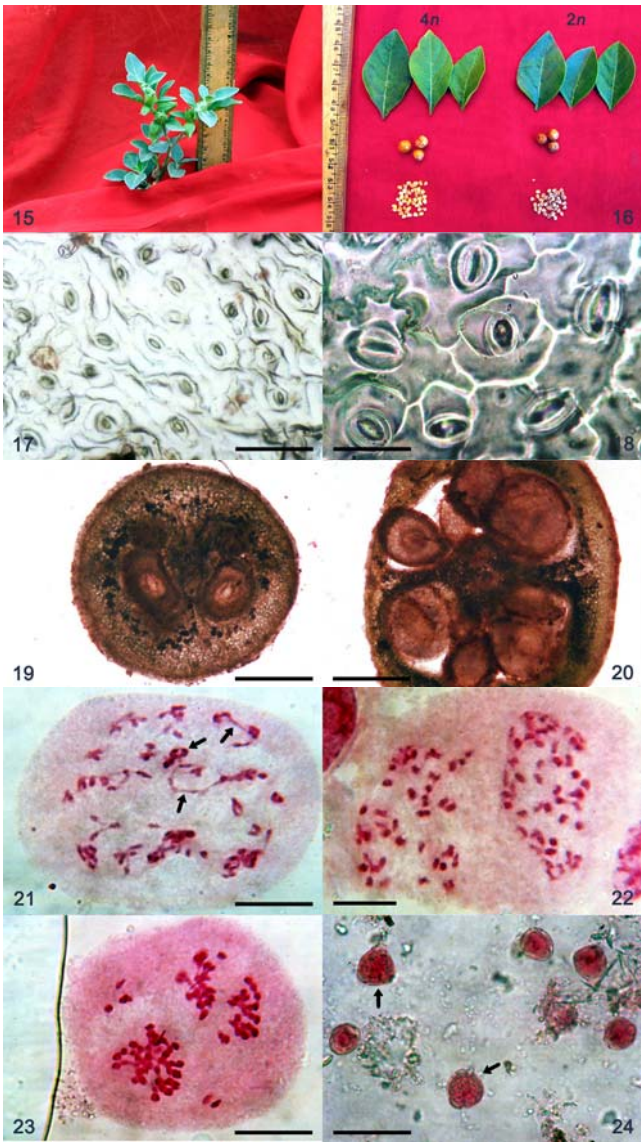

Figure plate III (**15-24**) *W. somnifera* var. 'Jawahar 22'. (**15**) Tetraploid plant at maturity; (**16**) Leaf, fruit and seeds of 4n and 2n plants; (**17-18**) Stomata of 2n (17) and 4n (18) plants; (**19-20**) Unequal sized ovule formation in tetraploid (scale bar = 1 mm); (**21**) 3IV+34II+16I (2n = 4x = 96) at MI of tetraploid; (**22**) AI showing 48/48 chromosome separation in tetraploid; (**23**) Irregular groupings and laggard formation at AI of the tetraploid; (**24**) Fertile (→) and sterile pollen grains in 4*n* (scale bar = 100 µm). **Figs. 21-23**: scale bar = 10 µm.

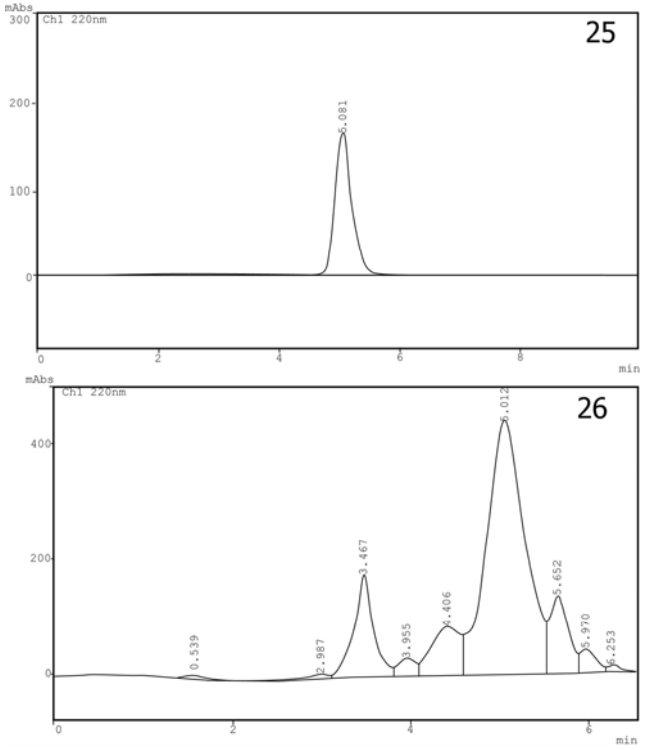

Figure plate IV (**25-26**) showing chromatograms of withaferin A. (**25**) Analytical standard; (**26**) tetraploid.

$P < 0.001$), seeds per capsule (DF 8, $P < 0.05$) and length of seeds (DF 18, $P < 0.001$) were found to be significantly higher in tetraploid than diploids (**Figs. 16-18**). Ovule sizes in tetraploid though not significant in relation to diploid, but was highly variable (length 21.32-36.15 μm^2 and breadth 17.14-26.45 μm^2). Due to paucity of flower buds only 2 scattered PMC's (3 IV + 34 II +16 I – **Fig. 21**; 3IV+35II+ 14I) could be analysed at MI; while, meiocytes at AI showed either 48/48 seperation of chromosomes (**Fig. 22**) or irregular distribution (**Fig. 23**). Frequency of pollen grains per microscopic field was significantly (DF 18, $P < 0.001$) lesser in 4n than 2n plants. Pollen fertility reduced in tetraploid (**Fig. 22**) than diploids; however pollen sizes did not show marked size variations. The tetraploids also showed significant enhancement in withaferin A content in leaves (DF 4, $P < 0.05$) than diploids (**Figs. 25, 26**) as esti-mated by HPLC. The seeds formed by the tetraploid failed to germinate in the following generation and furthermore new branches formed (after drying up of the 4n plant at maturity) from the retune were diploid as evidenced from cytological examination of flower buds.

Genetic variability analysis

Kandalkar (1988) studied 24 germplasms of *W. somnifera* and suggested that high heritability coupled with genetic advance were observed for number of berries on main stem, fresh weight of roots, total berries per plant and seed yield. Kandalkar *et al.* (1992) suggested additive and non additive gene effects for plant height, days to 50% flowering, shoot branches, root length and root yield per plant; while dominance effect was predominant with root yield. Complementary type of gene action was present for all traits analyzed. Kandalkar *et al.* (1993) from the study of genotypic and phenotypic correlations and path analysis were of opinion that high plant height and stem branches were useful criteria for improvement of root yield. Misra *et al.* (1998a) reported high heritability (broad sense) and genetic advance for plant canopy for *W. somnifera*. Misra *et al.* (1998b) studied 37 accessions of Ashwagandha based on 6 traits (plant height, plant canopy, leaf area, root length, root diameter and dry root yield) and reported that the materials represented 8 broad groups (cluster). Five accessions, namely, W.S.1, W.S.9, W.S. 20, W.S. 36 and W.S. 24 were widely divergent from each other as well as from the rest, and amongst them W.S.1, W.S.9, and W.S.24 were unique and consequently hybrids between them might be best for transgressive breeding. The accession for the same 6 traits were analyzed genetically by Misra *et al.* (1998a) analyzing 37 accessions for 6 important traits reported highest PCV (phenotypic coefficient of variation) and GCV (genotypic coefficient of variation) for dry root yield followed by plant canopy. Heritability and genetic advance were highest for plant canopy. Dry root yield was significantly and positively correlated with all component traits. Root diameter and root length made the highest direct and indirect contribution to root yield. Iqbal and Datta (2007d) reported that maximum contribution to seed yield was given by berries per plant followed by primary branches/plant and fresh weight of leaves/plant. Kumar *et al.* (2007) assessed 6 phenotypic markers and 3 withanolide markers in 25 germplasm of *W. somnifera* collected from different states of India for studying genetic variability using D^2 (statistical test) and PCA (principal component analysis) and observed distinct polymorphism grouped into 5 clusters.

Molecular genetics

Negi *et al.* (2000) investigated inter-and intra specific genetic genetic variation present in 35 individuals of *W. somnifera* using AFLP (amplified length polymorphism) marker technique and the information about genetic variation was employed to estimate similarity matrix value based on Jaccard's coefficient which was further used to construct a phenetic dendogram by UPGMA (unweighted pair group method of arithmetic averages). Two main clusters were detected with high level of polymorphism. Dhar *et al.* (2006) studied the genetic profile of 15 selected accession of *W. somnifera* using AFLP markers (64 primers were used). Among the primer used, 7 yield optimal polymorphism and a total of 913 polymorphic peaks were generated. Jaccard's similarity coefficient indicated that the accession possessed almost the same active compounds clustered together. Negi *et al.* (2006) studied the efficiency of selectively amplified microsatellite polymorphic loci (SAMPL) assay in assessing the levels of genetic diversity among *W. somnifera* genotypes and compared to standard AFLP technique and found SAMPL assay revealed higher levels of polymorphism than AFLP. Clusters analysis showed clear grouping within the *W. somnifera* Kashmiri and *W. somnifera* Nagori. One of the *W. somnifera* specific bands generated with SAMPL was used to develop a simple PCR (polymerase chain reaction) based assay and such diagnostic marker was used at the seedling stage to distinguish *W. somnifera* genotypes. Chaurasiya *et al.* (2009) studied RAPD, isozymes, polypeptide polymorphism and withanolides content in selected accessions of Indian *W. somnifera* in an attempt to cluster the accession to their characteristic profile. Results indicated widest phytochemical diversity among the Indian accessions. Senthil *et al.* (2009) reported expressed sequence tags (ESTs) in leaf and root (*in vitro*, 2-3-month-old sample) of *W. somnifera*, which generated 1047 leaf c-DNA and 1034 root c-DNA clones representing 48.5 and 61.5% unique sequence. The ESTs from leaf and root grouped into 239 and 230 clusters representing 22.8 and 22.2% of total sequence and of these 70% encoded proteins were found similar to characterize proteins from NCBI non-redundant data base with diverse molecular functions and biological processes based on gene ontology (GO) classification.

IN VITRO STUDIES

Embryogenesis

Rani *et al.* (2004) reported that somatic embryos were formed from calluses obtained from axillary shoots (raised

from nodal segments of glasshouse-grown plants under aseptic conditions), internodal segments (from in vitro raised plants), and root and cotyledonary leaf segments (from *in vitro* raised seedlings) after 8 weeks of initial culture. Embryo formation was highest (97.33%) from cotyledonary leaf callus on (MS) Murashige and Skoog (1962) medium containing kinetin (KN) (3 mg/l). Maximum of 66.67% cultures formed shoots on MS medium containing benzyl adenine (BA) (1 mg/l) in combination with indole acetic acid (IAA) (2 mg/l). The shoots raised from somatic embryos were rooted on MS medium supplemented with indole-3-butyric acid (IBA) (2 mg/l). The plantlets transferred to the field showed 70% survival rate after one year.

Micropropagation

Sen and Sharma (1991) observed shoot multiplication using low concentrations of BA (2.2, 4.4 and 8.9 uM). Maximum number of shoots (100-150 after 60 days) was obtained when 2.3 µM 2,4-dichlorophenoxy acetic acid (2,4-D) or 2.5 µM IBA was added to a medium containing 4.4 µM BA during the initiation of shoot multiplication. Kulkarni *et al.* (1996) developed shoots (using leaf explants) directly on MS basal medium supplemented with IAA and N^6-benzyl-aminopurine (BAP) and the shoots were easily elongated as well as rooted on MS media supplemented with 0.04 µM BAP. Plantlets were established in pots containing a mixture of sand: soil (1: 1 v/v) at a rate of 100%. Deka *et al.* (1999) showed that *in vitro* micropropagation can be achieved from axillary buds and shoot tips of *W. somnifera* on MS basal medium supplemented with BAP (0.1-1.0 mg/l) and KN (0.1-0.4 mg/l). Ghosh and Gupta (2001) found better shoot regeneration in half strength MS (containing 1 µM BA) and 0.3 µM 1-naphthalene acetic acid (NAA) and supplemented with the growth retardants triazole or Na-dikegulac at 1.6 or 0.4 µM) compared to axenic culture (lacking sucrose, vitamins and glycine) using nodal explant. Pawar *et al*. (2001) studied micropropagation and organogenesis in shoot tips and leaf-derived callus using MS medium with different plant growth regulators (PGR) and its combination (IAA, NAA, BA and KN) resulting on an average of 20-30 adventitious shoots development followed by rhizogenesis (90 ± 5: MS medium containing 4 µM IAA and 8 µM NAA) with post-transplantation survival rate of 70 ± 7%. Sabir *et al.* (2008) proposed an efficient method of *in vitro* shoot propagation methodology (MS medium supplemented with 1 mg/l BAP and 1 mg/l KN) in different accessions of *W. somnifera* and the raised shoots rooted easily in MS medium supplemented with 2 mg/l IBA and subsequently established in garden soil: vermicompost (3: 1, w/w) medium in a glasshouse. Withanolide content reported by the authors in micropropagated plantlets was lesser in comparison to field grown plants. The most likely reasons for the increased amount of withanolide were due to the age and maturity of the field grown mother plants, which allowed a greater accumulation of withanolides. However, Sabir *et al*. (2007) also demonstrated withanolide accumulation to vary due to the induction of different enzymes.

Callus development

Kulkarni *et al.* (1999) demonstrated direct regeneration of shoot buds in MS medium (either with BA or TDZ) from various explant sources (nodes, internodes, hypocotyls and embryos). The response varied for the explants used. Teli *et al.* (1999) found high frequency of callus induction from shoot tip explants (80%) compared with leaf explants (70%) on MS media supplemented with IAA (56 µM) and KN (56 µM). Kulkarni *et al*. (2000) induced multiple shoots from nodal (MS + 0.1-5.0 mg/l BA and 0.2-0.3 mg/l thidiazuron - TDZ), inter nodal (0.1-5.0 mg/l BA) and hypocotyl (MS + 0.5 mg/l BA) explants and those shoots were elongated and rooted (MS + 0.1 mg/l IBA or ½ strength MS lacking growth regulators) and finally acclimatized with 100% success rate except for embryo-derived plantlets (numerous shoot buds were produced from embryos, which could not be counted easily). Ray and Jha (2002) studied the efficiency of regeneration from somatic tissues of *W. somnifera* via organogenesis. The authors reported highest regeneration percentage (70-75%) from leaf explant. Addition of glutamine (50 mg/l) enhanced shoot proliferation and leaf growth and also increased the number of microshoots. Organogenesis was best under a 16-h photoperiod and BA (0.5 mg/l). The frequency of regeneration decreased in the dark. Plants regenerated by organogenesis were diploid and were free from any noticeable phenotypic variability. Cytological observations revealed 21 pairs of bivalent formation. Gaikwad and Prasad (2003) studied differential response of various culture media for callus induction and regeneration in Ashwagandha using cotyledon as explant. Of all the tested media, M3 medium containing 1 mg/l NAA, 2 mg/l BAP, 2 mg/l KN and 100 ml/l coconut milk proved to be the best. Govindaraju *et al.* (2003) developed high frequency plant regeneration protocol in Ashwagandha from internodal segment, leaf, root and petiole explants on MS and B5 media supplemented with 2,4-D (0.5-3.0 mg/l) and NAA (0.5-3.0 mg/l) either singly or in combination with KN (0.5-1.0 mg/l). Regeneration was observed in calluses of all the explants except roots on MS medium fortified with BAP (0.5-2.5 mg/l) singly or in combination with IAA (0.5 mg/l). Direct differentiation of multiple shoots from leaf, nodal segments and shoot tips occurred within two weeks on MS medium supplemented with BAP (0.5-3.0 mg/l) in combination with IAA (0.5 mg/l). Dwarf shoots were elongated on MS medium fortified with GA_3 (0.5 mg/l), rooted in half-strength MS media (both liquid and solid) with IBA (0.5-1.0 mg/l) alone in combination with IAA (0.5 mg/l) and plantlets were hardened for two weeks with 80-85% survival. Rani *et al.* (2003) reported maximum callus initiation (100%) in root and cotyledonary leaf segments grown in MS media supplemented with 2,4-D (2 mg/l) and 0.2 mg/l KN. Manickam *et al.* (2000) reported maximum callus proliferation on MS medium with 2.26 µM 2,4-D of which three-week-old, white, friable callus was used for shoot regeneration. Multiplication (8.4 ± 0.43 shoots per explant) of regenerated shoots was gained on MS medium supplemented with 4.44 µM of BA followed by rooting (5.1 ± 0.49 rootlets per shoot) of single shoots on half-strength MS medium supplemented with 9.84 µM of IBA and finally a hardening phase of 3 weeks. Siddique *et al.* (2004) optimized plant regeneration protocol using nodal explants in MS medium supplemented with 2,4-D, BA and KN (BA 1 mg/l and KN 2 mg/l induced highest callus formation). Sivanesan and Murugeson (2005) also developed an efficient plant regeneration protocol to induce plantlets using MS with 1.0 mg/l KN. Singh *et al.* (2006) standardized a protocol for plant regeneration from encapsulated shoot tips collected from in vitro proliferated shoots of *W. somnifera* using 3.0% sodium alginate and 75 mM $CaCl_2 \cdot 2H_2O$. The maximum percentage response (87%) for conversion of encapsulated shoot tips into plantlets was achieved on MS medium supplemented with 0.5 mg/l IBA after 5 weeks of culture.

Chemical analysis

1. Culture in liquid media

Ciddi (2006) established a suspension culture of *W. somnifera* to produce withaferin A (identificated by TLC, UV absorption, HPLC and electron spray mass spectroscopy) and the metabolite was strongly elicited by exposure to salacin (750 pM in amount to elevated withaferin A to 2.5 ± 2.9 mg/l compared to 0.47 ± 0.03 mg/l). Nagella and Murthy (2010) cell suspension cultures of *W. somnifera* were established in shake flask and the effect of growth regulators (auxin, combination of auxin and cytokinin), inoculum density (2.5-20 g/l), different media (MS, B_5, NN and N6), the strength of MS medium (0.25-2.0X), carbon source (sucrose, glucose, fructose, maltose), concentration

of sucrose (1-8%, w/v) and the initial pH (4.0-6.5) of the medium were determined for the production of withanolide A. The optimized conditions for biomass accumulation and withanolide A production were found to be 10 g/l of the inoculum on fresh weight basis, the full strength MS media, 3% (w/v) sucrose, four weeks culture period and the initial medium pH of 5.8. The investigation performed was important for scale-up process.

2. Culture in solid media

Undifferentiated callus cultures of *W. somnifera* failed to synthesize withanolides (Yu *et al.* 1974; Heble 1985). Wiermann (1981) attributed that varied capacity to synthesize secondary metabolites *in vitro* was due to morphological nature of explants used to initiate tissue culture lines. Roja *et al.* (1991) reported that callus cultures failed to synthesize withanolides, but multiple shoot cultures synthesized significant amounts, the concentrations were highest with 2.0 ppm BA + 10% coconut milk. Vitali *et al.* (1996) studied withanolide composition in *in vitro* cultures (MS supplemented with 0.5 mg/l BAP or 2.1 µM 2,4-D or free of plant growth regulators) and reported unlimited production in shoot and callus cultures. No withanolides were detected in hairy roots. Furmanowa *et al.* (2001a) revealed the presence of withaferin A from plantlets grown in greenhouse condition following the use of NMR and MS spectrophotometric method. Furmanowa *et al.* (2001b) further reported highest withaferin A content (700 µg/g dry wt) in winter cherry shoots grown for 5 weeks on NN medium supplemented with $ZnSO_4$ 0.0287 g/l compared with 400 µg/g DW in the control culture and after 7 weeks the production was more than 20% of the control value suggesting some adaptation to Zn^{2+}. Ray and Jha (2001) demonstrated regeneration and quantified withaferin A content in micropropagules. Sangwan *et al.* (2007) reported an amount of 2.38 mg/g DW of withanolide A from shoot culture and the extent of induction varied quantitatively (*ca*. 10-fold, 0.014 to 0.14 mg/g fresh wt) depending upon the PGR combination used as well as genotype. Sharada *et al.* (2007) suggested that organogenesis might be a key regulatory factor that stimulates production of withanolides *in vitro* and also pointed out that enzyme responsible for biogenesis of such compounds were possibly optimally operative in morphologically differentiated cultures. The authors also suggested that *in vitro* shoot tip explants contained the highest amount of withaferin A. Das *et al.* (2010) quantified the amount of withaferin A and withanolide A by HPLC under *in vitro* culture conditions in 3 different stages of callus development (stage I-undifferentiated callus; stage II and III-differentiated callus) on MS basal medium with dif-ferent hormonal (BA, KN, 2,4-D, IAA and IBA) combinations using epicotyl, shoot tip and leaf explants with the objective of developing a suitable protocol which ensure production of specific stipulated amount of secondary metabolites in a quick span of time. Results suggested that stage III of callus development yielded a significantly higher amount of alkaloids and withanolides including withaferin A and withanolide A irrespective of the nature of explant used.

Hairy root induction

Upon induction of hairy roots in *W. somnifera* using *Agrobacterium rhizogenes* (strain-LBA 9402/R1601)/*A. tumefaciens*-MTCC2250 (infecting shoots or leaf), a significant enhancement of secondary metabolites, specifically withaferin A and withanolide A and D was observed (Ray *et al*. 1999; Pawar and Maheshwari 2004; Kumar *et al*. 2005; Bandyopadhyay *et al*. 2007; Murthy *et al*. 2008). Pandey *et al*. (2009) described *A. tumefaciens* LBA 4404-mediated transformation in *W. somnifera* containing the binary vector pIG 121Hm along with Gus A reporter gene with intron under the transcriptional control of the *Cauliflower mosaic virus* (CaMV)33S promoter and found 1.67% transformation efficiency.

MARKET VALUE

The roots of *W. somnifera* is in great demand in the crude drug market and the annual Indian requirement is been estimated to be 9000 tonnes as per 2004/05 (PROTA 2008). The current farm gate price for the roots in India stands at US$ 1.50 per kg.

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