The genus *Withania* Pauquy belongs to the family Solanaceae which includes approximately 100 genera and 2500 species (Hunziker, 2001; Olmstead *et al*. 2008). The family has sub-cosmopolitan distribution with a major centre of diversity in Central and South America. In India, the family is represented by about two dozen genera and more than a hundred species of which 10 genera and 34 species are native (Deb, 1979). Among the worldwide list of twenty six species, the genus *Withania* is represented in India by *W. somnifera* and *W. coagulans*. A third species, *W. obtusifolia* Tackh was also reported from South India but according to Sundari *et al*. (1999) it is only a cytotype (2n = 24) of *W. somnifera* (2n = 48).

Within the family Solanaceae, *Withania* belongs to subfamily Solanoideae, tribe Physaleae and sub-tribe Withaninae of which it is the type genus (Olmstead *et al*. 2008). The generic name *Withania* commemorates the celebrated English ‘paleobotanist, ‘Henry Thomas Maire Witham’ with an orthographic variation of the final ‘m’ into an ‘n’ to which the commemorative termination –ia has been added. The specific epithet *somnifera* is a compound of two Latin words ‘somnus’ meaning sleep and ‘fero’ (ferere) meaning ‘to bear’. Thus the specific epithet alludes to sleep inducing properties of the plant. Species of the genus are represented by unarmed shrubs and perennial herbs with erect and much branched stems.

*W. somnifera* (L.) Dunal, the winter cherry, is an important medicinal plant. It has been in use in Ayurvedic and other systems of indigenous medicine for over 4,000 years (Anonymous, 1976). *W. somnifera* is an erect, branched, grayish, stellate-tomentose under-shrub with long tuberous roots. The Hindi name for the plant, ‘Ashwagandha’ means ‘smelling like a horse’. It is also called the ‘Indian ginseng’ for its restorative properties (Tripathi *et al*. 1996; Asthana and Raina, 1989; Singh and Kumar, 1998). Singh *et al*. (2000) compared the therapeutic properties of the roots of *W. somnifera* with those of the original ginseng (*Panax ginseng* C. Meyer) and found them similar in many aspects but different in their mode of action. However, Ashwagandha is not taxonomically related to the true ginseng; Ashwagandha belongs to the Nightshade Family, *Solanaceae* (of the order Solanales) whereas the original ginseng is a member of the Family, *Araliaceae* (of the order Apiales).
The species has a wide geographical distribution extending from southern Mediterranean region to the Canary Islands and to South and East Africa extending up to Sudan and Egypt. Further East, it is distributed from Palestine to North India ranging over Israel, Jordan, Iran, Afghanistan and Pakistan. In India, the species grows wild in the North Western region extending to an elevation of 1500 m in the hill regions of Punjab, Himachal Pradesh and Jammu and Kashmir (Singh and Kumar, 1998, Hunziker et al. 2001).

2.1. Medicinal properties

In Ayurvedic system of medicine, *W. somnifera* is claimed to have aphrodisiac, sedative, rejuvenating and life prolonging properties. It improves learning ability and memory capacity (Medharasayana). In the traditional medicine, ‘Ashwagandha’ is said to increase energy, youthful vigour, endurance, strength, health, vital fluids, muscle fat, blood, lymph, semen and cell production. It helps counteract chronic fatigue, weakness, dehydration, weakness of bones, loose teeth, thirst, impotence, premature aging, emaciation, debility, convalescence and muscle tension (Nadkarni, 1976; Asthana and Raina, 1989; Grandhi et al. 1994; Agarwal et al. 1999). It helps invigorate the body by rejuvenating the reproductive organs. The leaves of the plant are bitter in taste and are used as an anti-helmanthic. An infusion is given in fever. The list of curative and restorative uses of this plant has steadily increased and is growing by the day. Because of the large number of medicinal properties listed above, more than two hundred formulations sold in the market under different trade name have ashwangdha as the major component. Drug formulations from the roots have been found useful in constipation, rheumatism, nervous exhaustion and loss of memory (Singh and Kumar, 1998).

The modern and more recent properties attributed to *W. somnifera* include adaptogenic, anti-inflammatory, anti-oxidant, immunomodulatory, antimicrobial, radio-sensitizing, anti-stress, antidiabetic and anti-tumor activities (Devi et al. 1993, 1996; Agarwal et al. 1999; Bhattacharya et al. 1995, 2001; Davis and Kuttan, 2001, 2002; Dhuley et al. 2001; Owais et al. 2005). It has also been found useful in the treatment of bronchitis, mental illness, asthma, ulcers and arthritis (Ilayperuma et al. 2002). Pharmaceutical research and clinical trials have also brought out the usefulness of *W. somnifera* as a cure for anxiety, hepatoprotection and other cognitive and
Degenerative diseases such as Alzheimer’s and Parkinson’s (Ahmad et al. 2005; Kumar and Kulkarni, 2006; Gupta and Rana, 2007). The list of uses of different parts of this wonder plant is presented in Table 1.

2.2. Biochemical composition

The phytochemistry of *Withania somnifera* has been a subject of extensive study. Chemical characterization started with Power and Salway (1911) who isolated an amorphous alkaloid (C_{12}H_{16}N_{2}) from a South African strain of *Withania somnifera*. Later, Majumdar and Guha (1933) investigated a plant from Bengal (India) and confirmed the presence of the alkaloid. Later on, they reported presence of nicotine and seven other alkaloids from the roots which they named (without structural information) as sommiferine, somniferinine, somnine, withamine, pseudowithamine, withanmine and withanaminine (Majumdar, 1955). The chemical novelty and biological activities exhibited by these compounds triggered thorough chemical investigation of the plant resulting in the isolation and characterization of several compounds with similar structural features such as steroidal lactones, alkaloids, flavonoids and tannins (Lavie et al. 1975; Kirson et al. 1977, Eastwood et al. 1980; Atta-ur-Rahman et al. 1991, 1993; Kapoor, 2001).

The therapeutic value of *Withania somnifera* is ascribed to bioactive steroidal lactones called withanolides synthesized and accumulated in its leaves and roots (Sangwan et al. 2004a, b).

Withanolides are a group of naturally occurring C_{28} steroidal lactones built on an intact or rearranged ergostane framework, in which C-22 and C-26 are oxidized and make a six-membered lactone ring. Withaferin A (4β, 27-dihydroxy-1-oxo-5β, 6β-epoxywitha-2-24-dienolide) was the first member of this group of compounds to be isolated from a South-Asian strain. Till date, more than twelve alkaloids, forty withanolides and several sitoindosides (withanolides containing a glucose molecule at C-27) have been isolated and characterized from various parts of the plant (Kapoor, 2001; Anonymous, 2004; Kumar et al. 2007). Bandyopadhyay et al. (2007) have isolated and identified a few more withanolides. Compounds isolated from various parts of *Withania somnifera* are listed in Table 2.
2.3. Germplasm assessment

Evaluation of genetic diversity of crop species, as the one under study, is a pre-requisite for effective management of genetic resources, development of ‘core collections’ for monitoring genetic erosion, designing breeding and conservation strategies and for detection of contaminants and mixtures. Genetic diversity can be assessed by morphological plasticity or more directly by using molecular markers (Pejic et al. 1998). However, morphological traits are limited in number and are influenced by changes in the environment (Maric et al. 2004). Diversity estimates based on pedigree analysis, on the other hand, have also been found time consuming and unreliable (Fufa et al. 2005). Direct access to the genetic material by molecular markers provides an attractive alternative.

2.3.1. Morphological variability

Kaul (1957) reported that the cultivated morphotypes differ from the wild ones not only in their therapeutic properties but also in morphological characteristics of the root, stem, leaf, flower, pollen, mature fruit and seed. The author proposed a new species *Withania ashwagandha* for the cultivated morphotype. He argued that even in Persian literature the cultivated and wild plants are described separately as Kakanj-e-ebostani and Kakanj-e-jaballi respectively. Similar observations were made by Atal and Schwarting (1962) who reported a high degree of variability in *W. somnifera* with respect to growth habit and morphological characters. These authors identified five morphotypes from the Indian germplasm of *W. somnifera*. On pharmacognostic characterization, Sastry et al. (1960) and Dhalla et al. (1961a) also recorded significant differences between the wild and cultivated plants of *W. somnifera*. More recently, Kumar et al. (2007) studied morphological and chemical variations in *W. somnifera* germplasm and reported the distinct nature of wild accessions from the cultivated ones. They also reported that morphological and phytochemical variability existing in the germplasm was by and large gene controlled.

2.3.2. Biochemical variability

Abraham et al. (1968) reported three chemotypes of *W. somnifera* from Israel, each containing different steroidal lactones of the withanolide type. The authors did not report any morphological difference among the chemotypes even though they were
reported to have non-overlapping distribution. Withaferin A was reported to be the major compound in South African chemotype as also in the Indian chemotype I and Israel chemotype I (Abraham et al. 1968; Kirson et al. 1970, 1971) but it was reported absent from Israel chemotypes II and III (Abraham et al. 1968; Glotter et al. 1977; Nittala et al. 1981). There is evidence of more than one chemotype in India (Chakrabarti et al. 1974) but the exact number of chemotypes is yet to be ascertained. Chemotypes having several fold difference in the content of bioactive constituents have been reported from different parts of the world (Singh and Kumar, 1998). Scartzenni et al. (2007) reported that the Italian plants of this species were different from the Indian ones in withaferin A content. The authors employed six RAPD markers to mark genetic and related phytochemical differences between the Italian and the Indian types. Dhar et al. (2006) employed AFLP markers to study possible correlation of genetic data with the presence of secondary metabolites in *W. somnifera* chemotypes.

### 2.3.3. Molecular markers

Molecular markers have diverse applications in characterization of genetic variability (Karp et al. 1996). They have been utilized for assessment of genetic diversity in a large number of Solanaceous plant species like *Solanum tuberosum, S. melongena, Petunia* spp., *Nicotiana* spp. etc. Reclassification of plant families on the basis of DNA marker data is an example of its use in taxonomy (Olmstead et al. 1999). Since it is difficult to find an ideal DNA marker that meets all the criteria, a set of markers is commonly used for studies in genetic diversity (Weising et al. 1995; Robbinson and Harris, 1999). However, the choice of a particular molecular marker type depends upon the nature of the study and the degree of taxonomic resolution required (Zhang et al. 2000, 2002).

Bhutani (2000) reported that molecular markers can also be linked to genes of interest, allowing indirect selection of the desired genotype and also for the identification of commercial varieties. They are also useful in identification of the right chemotype to which therapeutic effects are attributed in a situation when there are more than one known chemotype as in the present case (Joshi et al. 2004). Chan (2003) emphasized upon the role of DNA markers as reliable tools for detection of genetic polymorphism as they reveal the unique genetic composition of each type and are not affected by
developmental and physiological conditions or by environmental factors. Morphological markers traditionally used in taxonomy and phylogeny suffer from the major disadvantage of being affected by character homologies (Scotland et al. 2003). These limitations have lent an added importance to molecular markers in resolving taxonomic problems (Bohs et al. 2005). However, the usefulness of combining morphometric and DNA markers’ data to solve taxonomic problems has been demonstrated in number of plant taxa (Shiran and Raina, 2001; Raina et al. 2001; Shipunov et al. 2004, 2005; Shah et al. 2008). Thus, DNA markers provide a precise method of germplasm assessment and characterization (Portis et al. 2005; Tam et al. 2005; Negi et al. 2006). In recent years, role of molecular markers in providing complementary evidence to conventional markers has been well documented (Spooner et al. 2006; Sehgal et al. 2008a,b,c,d; Nagpal et al. 2008; Sasanuma et al. 2008; Sehgal and Raina, 2008; Sesli et al. 2010).

2.3.3.1. Types of molecular markers

Various types of DNA-based molecular techniques are utilized to evaluate DNA polymorphism (Botstein et al. 1980; Powell et al. 1996). The available DNA markers can be categorized as: 1) hybridization based, 2) PCR based and 3) sequence based markers. These markers differ from each other in the level of detected polymorphism, dominant/co-dominant and (or) biallelic/multiallelic nature of the loci, ease of automation and reproducibility of the assay (Sehgal et al. 2008c).

2.3.3.2. Hybridization based markers

DNA profiles are visualized by hybridizing the restriction enzyme digested DNA to labeled probes. The probes could be low copy sequences derived from the genomic DNA, characterized/ uncharacterized cDNAs or specific gene probes of nuclear, mitochondrial and chloroplast genomes. RFLP markers have been used for a number of objectives in breeding viz., selection of traits of agronomic importance, quality testing of seeds and segregation analysis of progenies, evaluation of diversity of germplasm and for construction of linkage maps (Raina and Ogihara, 1995). RFLP polymorphism is a restriction pattern obtained due to point mutations such as single base pair loss or gain and indel events affecting the position and frequency of restriction sites in the DNA. RFLP is, however, expensive, time consuming and labour intensive. Hence it is not recommended when large number of samples have to be
analyzed. The available literature clearly indicates the lack of any RFLP study on *Withania somnifera*. However, RFLP has been used in taxonomic studies of several angiosperm families including Solanaceae. Prince *et al.* (1995) employed RFLP for assessment of inter-specific genetic variation within the genus *Capsicum* and pepper cultivars. Four main clades were reported in wild potatoes on the basis of RFLP data of chloroplast DNA (Spoonier and Castillo, 1997).

### 2.3.3.3. PCR-RFLP and Sequencing

A modified version of RFLP is PCR-RFLP, also called Cleaved Amplified Polymorphic Sequences (CAPS) wherein a PCR locus is first amplified with PCR and then subjected to restriction enzyme digestion. Such digests are compared for their differential migration in electrophoresis (Jarvis *et al.* 1994). PCR primer for this process can be synthesized from the sequence information available in databank of genomic or cDNA sequences or from cloned RAPD bands.

Internal transcribed spacer (ITS) regions of the nuclear rDNA genes ITS1 and ITS2 have proved to be informative regions of variable DNA sequences for analysis of species relationships in a number of angiosperm genera (Baldwin *et al.* 1995; Sang *et al.* 1995; Wendel *et al.* 1995; Clements, 2003; Orthia *et al.* 2005). The ITS regions are non-coding DNA sequences that are transcribed to RNA, but spliced out during ribosome assembly. Being non-coding, they accumulate mutations much more rapidly than the 5.8S rDNA gene. On the other hand, ribosomal genes, although present in high copy number, have usually homogeneous DNA sequences within individuals. As such they are essentially equivalent to single gene loci. Concerted evolution is cited as the mechanism for such homogeneity of DNA. And wherever concerted evolution is incomplete, multiple nuclear rDNA sequence types are present in the genome (Zhang and Sang, 1999). Thus variations in such loci have a high power of discrimination (Weider *et al.* 2005). The nuclear genomic ITS1-5.8S-ITS2 regions of rDNA are contiguous and can be readily amplified by PCR as a single unit.

Ando *et al.* (2005) resolved phylogenetic relationships within the solanaceous genus *Petunia* by chloroplast DNA RFLP using nineteen restriction enzymes and twelve hybridization probes from *Nicitiana* chloroplast DNA. The authors concluded that the genus *Petunia sensu* Jussieu includes the sister clades of *Petunia* and *Calibrachoa sensu* Wijsman and that speciation in the genus has occurred independently in several
different regions and is comparatively recent. Chloroplast primers with trnH-psbA were successfully used by Kress et al. (2005) in plant barcoding. Whipple et al. (2007) also employed three chloroplast DNA intergenic regions viz, trnK-rps16, trnC-petNlr and trnH-psbA in distinguishing North American species of the grass genus *Glyceria*.

In the past two decades, sequencing of genes has provided a comprehensive body of data an evidence for reconstruction of plant phylogenies. Rapid accumulation of data on gene sequences in the databases and the advances made in cloning techniques have greatly contributed in this effort. The structural organization of many genes viz., ribosomal RNA gene (18S-5.8S-26S) clusters from nuclear as well as organelle genomes, genes for synthesis of larger subunit of rbcL located in chloroplast genome, gene clusters for the synthesis of 5S ribosomal RNA, gene for synthesis of ATP subunit located in mitochondrial genome are such that it has led to design of universal primers for their amplification across angiosperm taxa. The nuclear genomic ITS1, 5.8S and ITS2 regions of rDNA are contiguous. They have a total length of ~700 bases and can be readily amplified by PCR as a single unit and studied as a single construct. A number of other regions of DNA such as trnK of chloroplast and spacer region of 5S rDNA have been sequenced as diagnostic tools for characterization and authentication of species. Data on DNA sequencing together with computer-aided cladistic analysis have contributed significantly to the understanding of relationships of flowering plants (Kitching et al. 1998). Ribosomal DNA gene families are constituted by conservative, partly conservative and non-conservative regions. Some other genes, as listed above, also exhibit variations that help in constructing phylogenies (Fairfield et al. 2004).

Apart from phylogenetic analysis, DNA sequencing data has been used as a definitive method for species identification. Variations due to transversions, insertions or deletions can be assessed directly and the information on a pre-defined locus can be obtained. Extensive genetic variation occurs at single nucleotide level. Direct sequencing can help identify such single nucleotide polymorphisms that usually depend on how closely related are the organisms being compared.

**2.3.3.4. PCR based markers**

PCR based molecular markers include RAPD, ISSR, SSR, PCR-RFLP and AFLP. They have been recommended for intra-specific and inter-specific studies in biodiversity assessment and taxonomy (Escaravage et al. 1998; Hodkinson et al. 2002a,
2002b). These markers involve in-vitro amplification of defined DNA sequences or loci with the help of specific or arbitrary primers and the thermostable DNA polymerase.

**(a) Randomly Amplified Polymorphic DNA:** Among the different DNA markers, RAPD markers have been frequently used for genetic analyses (Williams *et al.* 1990). Owing to simplicity, efficiency and non requirement of sequence information, RAPD has been employed as a marker of choice for taxonomic studies in *Solanum melongena* (Karihaloo *et al.* 1995). Spooner *et al.* (1995) showed the applicability of RAPDs for both intra and inter-specific studies in section *Eutuberosum* of the genus *Solanum*.

RAPD has been employed in taxonomic and phylogenetic studies in some members of the family Solanaceae (Bogani *et al.* 1997; Rodriguez *et al.* 1999; Bamberg and Rio, 2004). RAPD markers helped to distinguish heterogenous populations of wild potato from closely related tetraploid species, *S. oplocense*, *S. gourlayi* and *S. tuberosum*, which suggests its taxonomic isolation (Bamberg and Rio, 2004). In addition to phylogenetic reconstruction, RAPD markers have also been utilized in the detection of DNA sequence polymorphism and isolation of markers linked to various traits. Singh *et al.* (2006) used RAPD as a tool for assessing genetic diversity and species relationships among twenty eight species and accessions of the genus *Solanum*. It discriminated all the accessions and the resulting dendrogram showed that *Solanum incanum* was closer to *S. melongena* than to *S. nigrum*. Suganda *et al.* (2006) applied RAPD to distinguish three cryptic Indonesian species of *Brugmansia*, which, were indistinguishable at the morphological level. Each species was found to have a different DNA fragment pattern, thus allowing their genetic characterization. The division of the genus *Hippophae* into two sections has been supported by RAPD fingerprinting at inter- and intra-specific levels (Lian, 1988; Sheng *et al.* 2006). Scartzeni *et al.* (2007) differentiated the Indian and Italian strains of *W. somnifera* on the basis of RAPD fingerprinting data. Sesli *et al.* (2010) has employed RAPD markers together with morphological characteristics to differentiate the eighteen cultivated olive strains. Ahlawat *et al.* (2010) have also employed RAPD markers for genetic analysis of *Acorus calamus* and distinguished the diploid and tetraploid accessions into two separate groups. Notwithstanding their potential to unravel intra-specific variability, RAPD markers are often avoided for poor reproducibility of
results mainly due to generation of unreliable bands and varied levels of amplification between reactions complicating data interpretation (Smith et al. 1994; Thorman et al. 1994).

(b) **Inter Simple Sequence Repeat:** ISSR is a specific primer-based polymorphism detection system, where a terminally anchored primer specific to a particular simple sequence repeat (SSR) is used to amplify the DNA between two opposed SSRs of the same type that falls within the amplifiable range (Zeitkiewicz et al. 1994). Such amplification does not require genome sequence information and leads to multi-locus and highly polymorphic patterns (Nagaoka and Ogihara, 1997). Like RAPD, ISSR markers are quick and easy to handle, but they have higher reproducibility because the primers have a longer sequence of bases. Polymorphism occurs whenever one genome is missing in one of the SSRs or has an indel or any other chromosomal aberration (Wolfe et al. 1998). In the Solanaceae, ISSR markers have been used mainly for cultivar identification (McGregor et al. 2000). Kumar et al. (2001) used ISSR markers successfully for classification and identification of taxa in *Capsicum annuum*. SSR motifs exist in multiple copies in eukaryotic genome with some of them oppositely directed in vicinity (Li and Ge, 2001).

(c) **Amplified Fragment Length Polymorphism:** The AFLP marker system which combines the efficiency of PCR with the specificity and reliability of RFLP was developed by Vos et al. (1995). This technique and its modified version named as SAMPL (Selectively Amplified Microsatellite Polymorphic Loci) are used to visualize hundreds of amplified DNA restriction fragments simultaneously. These fragments are produced by restriction digestion and amplification using PCR, followed by analysis by autoradiography or by the use of fluorescently labeled primers. Total genomic DNA is digested with restriction endonucleases (a frequent cutter and a rare cutter) followed by ligation of double stranded DNA adaptors to the digested genomic DNA. Pairs of oligonucleotide primers complementary to the adaptor sequences and with one to four additional 3’ nucleotides are utilized for PCR. The polymorphism in the DNA fingerprints arises from alteration in the DNA sequences including point mutations which abolish or create restriction sites and insertions, deletions and inversions between two restriction sites (Zabeau, 1993; Vos et al. 1995).
AFLP is currently being used as a molecular marker technique for the construction of linkage maps, marker assisted breeding and molecular typing. AFLP primers were found to be four times more efficient than RAPD primers in the detection of DNA polymorphism in *Capsicum annuum* a member of the family Solanaceae (Paran *et al.* 1998). AFLP has been used in the analysis of genetic diversity in crop plants because of a wide coverage of the genome with a single reaction in a short time and production of more polymorphic loci per primer than SSR or RAPD for DNA of any origin and complexity (Bogani *et al.* 1997; Kumar, 1999). Moreover prior knowledge of the genome is not required. For these reasons, AFLP finds favour for diversity analysis of plant systems like the one under study, where the genome is largely unexplored (Ridout and Donini, 1999; Hodkinson *et al.* 2000). Negi *et al.* (2000) investigated genetic variation at inter- and intra-specific level in some Indian accessions of *W. somnifera* using four AFLP markers. They employed 32 accessions collected from different parts of the country. The authors identified two varieties namely Nagori and Kashmiri. Ren and Timko (2001) used AFLP markers for polymorphism analysis and evolutionary relationships among cultivated and wild species of the Solanaceous genus *Nicotiana*. The technique was also used for inter-population studies and also to establish relationship between different species or sub-species (Amsellem *et al.* 2000; Zhang *et al.* 2001; Despres *et al.* 2003). AFLP has also proved to be an efficient tool in the identification of diagnostic or specific markers (Campbell *et al.* 2003). The amplification of maximum number of polymorphic bands for each primer from DNA of any origin has made these markers a preferred choice for analysis of genetic diversity (Campbell *et al.* 2003; Despres *et al.* 2003). In recent years AFLP and its modified version SAMPL have become the preferred techniques for detection and quantification of genetic polymorphism in plant species (Bahulikar *et al.* 2004; Negi *et al.* 2006). Julio *et al.* (2005) developed AFLP markers linked to three disease resistant varieties in *Nicotiana tabacum*. The efficacy of SAMPL and AFLP assays in assessing genetic diversity among *W. somnifera* genotypes was tested by Negi *et al.* (2006) to discriminate the Kashmiri genotypes from the Nagori genotypes. These two groups clearly discriminated at a low similarity value, indicating that these two groups were highly divergent. Dhar *et al.* (2006) reported a high degree of congruence between the secondary metabolites and AFLP markers by studies on fifteen accessions of *W. somnifera* selected from various parts of India. AFLP has been employed for
determining genetic relationships among several angiosperm taxa (Sehgal et al. 2005, 2008a, b). Recently Kwon et al. (2009) successfully obtained two species specific SCAR markers for *Panax notoginseng* from AFLP derived DNA fragments and established the identity of this species in the genus *Panax*. AFLP markers have also been used by Esfahani et al. (2009) to investigate the level of genetic diversity within and among different geographical groups of potato varieties of Iran. More recently, Shaheen et al. (2010) also utilized AFLP for genetic diversity and phylogenetic analysis of the family Malvaceae.

### 2.4. Reproductive biology

Reproductive biology of Indian accessions of *W. somnifera* has been investigated from time to time (Ram and Kamini, 1964, 1965; Garg and Bhatnagar, 1988). A lot of work on reproductive biology and breeding system of medicinal plants has been reported (Ramirez and Brito, 1990; Talavera et al. 1993).

#### 2.4.1. Floral biology

Despite their relevance in cultivar improvement and conservation of the species, less attention has been paid to floral biology, breeding system and pollination mechanism of *W. somnifera*. It produces flowers indeterminately round the year with a peak of flowering between March and July. Floral architecture of *W. somnifera* is similar to other members of Solanaceae. The flowers born in axillary clusters, are pedicellate, ebracteate, yellowish-green and hairy. Calyx is gamosepalous; enlarged and inflated in fruit as the fruiting calyx; sepals are 5-partite, persistent with linear acute lobes, tomentose outside and glabrous inside. Corolla is 5-lobed and gamopetalous, the lobes spreading or recurved, acute, pubescent and greenish-yellow. Reproductive apparatus comprises five epipetalous stamens and a single pistil. Stamens are attached near the base of the corolla. Each stamen bears a long filament terminating in a bilobed anther. The pistil comprises of a proximal glabrous ovary and an erect papillate stigma. The ovary is bilocular and the placcentation axile. The excess flowers are born simply to fulfill the male function and fruits are aborted to adjust the number to available resources (Bawa and Webb, 1984; Lloyd, 1980).

The species exhibits stigma-anther proximity caused by elongation of filaments to cover the bilobed stigmatic surface with dehiscing anthers. It has been reported that the
mechanism of pollination in *Petrocoptis viscosa*, an herb endemic to the North-West Iberian Peninsula is facilitated by similar flower morphology and by reflexing of stigma to contact its own anthers (Navarro and Guitian, 2002). High pollen load on the stigma and stiff pollen competition within a flower strongly favours self-pollination (Primack, 1985).

### 2.4.2. Breeding system

Apart from floral architecture, plants possess a number of physiological mechanisms that influence mating patterns, particularly the degree of self fertilization (Bertin and Newmann, 1993; Eckert and Barrett, 1994; Griffin *et al.* 2000; Gituru *et al.* 2002). Floral and reproductive features create a syndrome of characters favouring autogamy, geitnogamy, xenogamy or a blend in some proportion (Darlington, 1958; Stebbins, 1974; Sharma *et al.* 1992b). Plant species display a whole range of amphimictic systems from obligate selfing to obligate out-crossing (Darlington, 1958; Lalonde and Roitberg, 1994). Work on sex expression and reproductive behaviour of *Withania* is scanty. However Kaul, *et al.* (2005) have studied some aspects of the reproductive biology of *W. somnifera*, Anderson *et al.* (2006) has reported the breeding behavior of dioecious species *W. aristata*. Recently, Latoo *et al.* (2007) reported their findings of polen-ovule ratios and the reproductive success of the species.

### 2.4.3. Resource allocation

Reproductive strategies for maximizing the seed output from a given amount of reproductive effort were discussed by Lloyd (1980). Thus the pattern of resource allocation constitutes an important aspect of plant reproductive strategies (Primack and Antonomvics, 1982; Dunn and Sharitz, 1991). Plants utilize available resources for growth, maintenance and reproduction in a manner which maximizes their reproductive output. Reproductive resource allocation is the proportion of energy allocated for reproductive functions in relation to the total energy available to the individual. The patterns of reproductive effort and resource allocation have been studied in some other plants of medicinal importance. Sharma and Kaul (1995) made a detailed analysis in *Plantago ovata* and its wild ally, *P. major*. 

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2.4.4. Development

Flowers in *W. somnifera* bear five epipetalous stamens with long filaments and bilobed, dorsifixed anthers which dehisce longitudinally. The ovary is superior, bilocular with 30-45 ovules, borne on massive axile placentae. The style is long and the stigma capitate. Pollen grains are shed at two celled stage. On the day of anthesis, the embryo sac is organized and the egg is ready for fertilization. Pollination takes place on the day of anthesis and fertilization is accomplished the next day. Embryo sac development is *Polygonum* type and embryo development is solenoid type (Bhaduri, 1936; Ram and Kamini, 1965). Zygote divides between the second and fourth day after anthesis. Quadrant polyembryos are formed on the 6th day and by the 8th day, embryo attains the globular shape. On the 12th day after anthesis, the embryo becomes heart shaped followed by a mature horse shoe shaped embryo on the 18th day (Ram and Kamini, 1964, 1965). After fertilization the volume of the ovary remains constant for the first two days and attains maximum volume on the 14th day. The maximum weight of the fruit has been recorded on the 28th day after anthesis. It was reported that ovaries were light green in colour up to sixth day after anthesis and start developing red colour on 22nd day due to disappearance of chlorophyll from the pericarp. Dark reddish-brown colour was developed by the 28th day (Ram and Kamini, 1965).

2.4.5. Cytogenetic studies

The species has been reported to show ploidy level variations. With a haploid number of twelve, the diploid, $2n = 24$ (Ram and Kamini, 1964), tetraploid, $2n = 48$ (Bir and Sidhu, 1980) and hexaploid, $2n = 72$ (Bir and Neelam, 1984) cytotypes have been reported from different parts of the country. Ray and Jha (2002) reported twenty one regular bivalents in regenerated plants of *W. somnifera*. Karyotype analysis of the species revealed seven groups of chromosomes with prevalence of metacentric and sub-metacentric types. The species is also reported to show polysomatomy ($2n= 12, 2n= 18, 2n= 24, 2n= 36, 2n= 48$ and $2n= 72$) with a predominance of $2n= 48$ type (Iqbal and Dutta, 2007). Karyotype was reported to be symmetric with chromosome length varying from 1.43 µm to 2.64 µm (Iqbal and Dutta, 2007; Lattoo *et al.* 2007).

Meiocytes show twenty four bivalents at metaphase I with some bivalents having a rod configuration. There is a persistent occurrence of secondary association of bivalents which points towards polyploid nature of the species. The species seems to have
undergone diploidization in the course of evolution resulting in regular meiotic behaviour (Iqbal and Dutta, 2007). Recently, cytological investigations carried out in four different populations revealed the existence of diploid and tetraploid cytotypes in the species and the phenomenon of cytomixis was cited as the probable reason for cytological variation (Singhal and Kumar, 2008).

2.5. Environmental stress

Temperature stress is known to cause physiological, biochemical and molecular changes resulting in protein denaturation, lipid liquefaction and perturbation of membrane integrity (Levitt, 1980). Exposure of plants to low temperature causes increased production of reactive oxygen species (Bowler et al. 1992). Various biochemical and physiological effects including those on photosynthesis, growth and development are imputed to enhanced levels of Reactive Oxygen Species (Wise, 1995). Reactive oxygen species include superoxide radicals (O$_2^\cdot$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radical (OH$^\cdot$) which are produced in the cell, particularly in chloroplasts and mitochondria (Mittler, 2002). ROS have been reported to cause oxidative damage to proteins, lipids and DNA affecting normal functions of the cell (Foyer and Fletcher, 2001; Misra and Gupta, 2006). Iqbal et al. (2006) also reported that increased ROS production in plants on exposure to abiotic stresses affects photosynthesis and other metabolic functions.

Plant cells synthesize a variety of antioxidants to neutralize the ROS produced under normal and stress conditions. Major ROS scavenging agents include complex non-enzymatic antioxidant molecules viz., ascorbic acid (AA), reduced glutathione (GSH), α-tocopherol (α-toc), and antioxidant enzymes like catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR) and superoxide dismutase (SOD) (Jaleel et al. 2007b). Accumulation of secondary metabolites beyond certain concentrations is often taken as an indicator of stress injury in the plant.

Perusal of literature showed a few articles on water and salt stress but a complete lack of information on the responses of *Withania somnifera* to low temperature stress. Jaleel et al. (2008f) carried out the salinity stress studies in *Withania somnifera* and observed that triadimefon (TDM) treatment ameliorated the injurious effects of sodium chloride stress and led to an increase in growth and synthesis of photosynthetic pigments, non-enzymatic antioxidants and antioxidant enzymes. Water stress also significantly
increased activity of antioxidative enzymes like SOD, APX and CAT in *W. somnifera* (Jaleel, 2009). It is now generally accepted that environmental stress triggers a wide variety of plant responses, ranging from changes in plant metabolism and growth rates to alterations in gene expression (Jaleel, 2009).

2.6. *In vitro* studies

2.6.1. Regeneration

*In vitro* cultures have been extensively utilized for fast propagation of elites, hybrids and transgenics. It enables mass and rapid multiplication of the desired genotypes under controlled conditions.

Protocols for *in-vitro* propagation of *W. somnifera* through androgenesis were developed by Vishnoi *et al.* (1979). Similarly, a protocol for multiple shoot formation from axillary buds was developed by Roja and Heble (1991). Sen and Sharma (1999) obtained shoot multiplication from shoot tips and nodal explants of aseptically germinated seedlings of *W. somnifera* by using low concentrations of BA. Direct plant regeneration and shoot multiplication have been reported from explants obtained from leaf, nodes, internodes, hypocotyl, and embryos of the selected chemotypes (Baburaj and Gunasekaran, 1995; Kulkarni *et al*. 1996; Kulkarni *et al*. 2000). Indirect shoot regeneration from internodal explants was employed for propagating *W. somnifera* (Manickam *et al*. 2000). Multiple shoot cultures of *W. somnifera* from single shoot tip explants have also been reported by Ray and Jha (2001). The authors recorded twenty seven fold increase in the number of micro shoots by reducing agar concentration to 0.16 %. Saritha and Naidu (2007) used axillary bud and nodal explants for micro-propagation of *W. somnifera* and for inducing *in-vitro* flowering and fruiting. *In-vitro* fruiting was obtained on MS medium supplemented with Kn (2.0 mgl$^{-1}$) and IAA (0.1 mgl$^{-1}$). Nodal explants of *W. somnifera* differentiated shoot buds on three different media viz., MS, SH and B$_3$ containing different concentrations and combinations of auxins and cytokinins (Sivanesan and Muruqesan, 2008). An efficient protocol for indirect regeneration from internode and leaf explants on MS medium supplemented with BAP and IAA has been developed by Dewir *et al*. (2010).
2.6.2. Withanolide production

Roja and Heble (1991) analyzed in-vitro grown tissues for withanolide biosynthesis. Shoot cultures contained withanolide I, G, and D while unorganized callus culture were reported to be deficient in withanolides. Similarly, Rani and Grover (1999) also reported that callus and suspension cultures derived from Withania spp. lack measurable quantities of withanolide in the undifferentiated cultures. Leaf extracts of the tissue culture raised plants did not show immunosuppressive activity possibly due to the absence of withanolide A (Furmanowa et al. 2001).

Ray and Jha (2001) investigated production of withaferin A and withanolide D in multiple shoot cultures of *W. somnifera*. Shoot tips cultured on MS medium supplemented with BA (1 mg l\(^{-1}\)) induced about ten micro shoots per explant and showed the accumulation of withaferin A (0.04 %) and withanolide D (0.06%). The culture showed enhanced accumulation of both withaferin A (0.16%) and withanolide D (0.08 %) when the MS solid medium was supplemented with BA (1.0 mg l\(^{-1}\)) and 4% sucrose. However when MS medium was supplemented with BA (1.0 mg l\(^{-1}\)) and 10% coconut milk, twenty seven fold increase in biomass was recorded. Multiple shoot cultures of two selected experimental lines (*RS-Selection-1* and *RS-Selection-2*) were developed from axillary buds on MS medium supplemented with BAP and Kn. Subsequently the cultures were analyzed for concentration of withanolide-A by HPLC (Sangwan et al. 2004).

Direct rooting from leaf explants of *W. somnifera* has been achieved by Wadegaonkar *et al.* (2006) on half-strength MS medium supplemented with sucrose at 15 gl\(^{-1}\) and several concentrations and combinations of growth regulators. Roots so obtained and cultured on MS liquid medium for establishment of root-organ cultures showed higher concentration of bioactive constituent as compared to roots of field grown plants.

Ciddi (2006) reported detectable levels of withaferin A and withanolide A from unorganized cultures. *In-vitro* synthesis of bioactive withanolides from multiple shoot and root cultures from direct rhizogenesis in leaves, callus tissue and single cell suspension cultures of *W. somnifera* has been achieved by several workers (Sangwan *et al.* 2005; Sabir *et al.* 2007a, b.). This has opened avenues for large scale *in-vitro* production of these active principles. The hormonal combination of BA and Kn has been reported to induce morphogenic response and the biogeneration of withaferin A.
and withanolide A in the *in vitro* cultured shoots (Sangwan *et al.* 2007). Withanolide production requires a level of organ differentiation for biosynthesis and accumulation as seen in case of phytochemicals of the ginseng (Liang and Zhao, 2008). However Sabir *et al.* (2008a, b) reported withaferin A and withanolide A from undifferentiated callus cultures as well.

Sharada *et al.* (2007) reported that production of withanolides is closely associated with morphological differentiation. Withaferin A and withanolide A have been reported from the regenerated tissues (Sabir *et al.* 2008a). More recently, Ahuja *et al.* (2009) isolated four glycowithanolides viz. withanoside IV, withanoside VI, physagulin D and withastraronolide from multiple shoot cultures of *W. somnifera*. Apart from production of withanolides, *in-vitro* cultures provide a method to understand their biosynthetic pathway in cell, organ and tissues cultures under controlled conditions.

### 2.7. Genetic transformation

In medicinal plants, there are very few reports of genetic transformation by direct introduction of desirable genes altering metabolic pathways that regulate secondary plant products. The often-cited reason is that these plants have characteristic metabolic pathways which hinder integration and expression of foreign genes (Saito *et al.* 1992). However, the application of systems biology in unraveling the gene regulatory circuits in relation to bioactive components holds some promise.

Banerjee *et al.* (1994) carried out hairy root transformation of *W. somnifera* by three different strains of *Agrobacterium rhizogenes* (A4, LBA 9402 and LBA 9360) and analyzed the specificity of withanolide production potential with special reference to withaferin A. The best response in terms of transformation ability, growth of the hairy roots and withaferin A production was obtained with strain A4, followed by LBA 9402. However, the strain LBA 9360 failed to induce the transformation event. Maximum production was reported from hairy roots of 10-week-old cultures. *In vitro* withanolide production in roots transformed by *A. rhizogenes* (derived from the Sicilian plant) and supplemented with BA or 2, 4-D, was investigated by Vitali *et al.* (1996). Withanolide production by hairy root cultures of *W. somnifera* transformed with *A. rhizogenes* (LBA 9402) has also been reported by Ray *et al.* (1996). Hairy roots grown on MS medium containing 3% (w/v) sucrose in the absence of exogenous
plant growth regulators synthesized several withanolides from which withanolide D was isolated and identified. Withanolide D production was higher in the transformed than in the untransformed root cultures (Ray et al. 1996). Hairy root culture system aided by *A. rhizogenes* has become a popular method of secondary metabolite synthesis (Palazón et al. 2003b). The hairy root phenotype is preferable for its fast growth, genetic stability and independence from geotropic asymmetries and lateral branching.

Transformation of *W. somnifera* with wild type nopaline and octopine strains of *A. tumefaciens* was reported by Ray and Jha (2001). The oncogenic strains showed different levels of virulence in the two genotypes studied, differing mainly in the nature of the galls formed and in their subsequent morphological competence. Ten percent of the galls obtained after infection with nopaline strain N2:73 spontaneously developed shooty teratomas of an altered phenotype. These teratomas grew in unsupplemented basal medium and were able to synthesize both the major withanolides of the parent plants. Withanolide synthesis in shooty teratomas was much higher (0.07–0.1% withaferin A and 0.085–0.025% withanolide D) compared to ordinary shoot cultures. Secondary metabolites produced by hairy roots arising from the infection of plant material by *A. rhizogenes* were reported to be quantitatively the same as those synthesized in intact parent roots but superior in certain cases (Sevon and Oksman, 2002).

Bandyopadhyay et al. (2007) reported the presence of TR-DNA in the transformed callus lines of *W. somnifera* obtained after infection with *A. rhizogenes* strain A4, thus confirming the effects of aux genes on root line phenotypes. The authors reported that variability in development and withanolide production was introduced into *W. somnifera* by different strains of *A. rhizogenes*. Accumulation of withaferin A was maximum (0.44% of the dry weight) in transformed hairy root lines. All the rooty callus lines accumulated both withaferin A and withanolide D. The authors also reported that some of the callus lines also produced both withaferin A (0.15–0.21% dry weight) and withanolide D (0.08–0.11% dry weight), and that they grew faster than the transformed root lines. Chaudhury et al. (2009) have shown transfer of a synthetic gene encoding for a fungal cryptogein in *W. somnifera*. This cryptogein developed resistance in the transformed plants against the fungus. In addition, increased
transgenic mimicry resulted in an increase in secondary metabolites and biomass production in transformed root cultures of *W. somnifera* (Mijalali *et al.* 2009).

**2.8. Biosynthetic pathway of withanolides**

It has been reported that, except for few exceptions, the plants that synthesize the 20-H withanolides are unable to produce the 20-OH counterparts and vice-versa (Kirson *et al.* 1977). The biosynthetic pathways of withanolides and other chemical constituents of *W. somnifera* are not fully known, and a very little information is available on their biogenetic aspects (Kirson *et al.* 1977; Nittala and Lavie, 1981; Ray and Gupta, 1994). However, the available information has been pieced together to propose a tentative scheme. Withanolides are probably derived from cholesterol which is the starting material for their biosynthesis. The first step is the activation of acetate by its conversion to acetyl Co-enzyme A. Two units of acetyl CoA are combined and metabolized to mevalonic acid. Only the R- form of mevalonic acid is used by the living system to produce terpenes, while the S- form is metabolically inert. The (R)-mevalonic acid is converted into isopentenyl pyrophosphate (IPP) through the loss of one carbon atom. One molecule of 3-isopentenyl pyrophosphate (IPP) can condense in a head-to-tail manner with its isomer, 3, 3-dimethyl allyl pyrophosphate (DMAPP), to yield geranyl pyrophosphate (GPP). A condensation reaction of trans geranyl pyrophosphate with another molecule of IPP yields farnesyl pyrophosphate (FPP). The enzyme squalene synthase catalyses the condensation of two molecules of farnesyl pyrophosphate in a head-to-head manner in the presence of NADPH to produce squalene. Oxidation of squalene by atmospheric oxygen is catalyzed by NADPH-linked oxide to result in squalene 2, 3-epoxide. The latter undergoes ring closure to form lanosterol which is then converted into a variety of different steroidal triterpenoidal skeletons. However, the bioconversion of lanosterol to 24-methylencholesterol is not fully understood. The sequence of reactions and intermediates may also differ among organisms. 24-Methylenecholesterol may be a biosynthetic precursor of steroidal lactones. It has been proposed that the hydroxylation in C22 and δ-lactonization between C22 and C26 of 24-methylencholesterol yields withanolides.

Thus, withanolides are metabolic descendants of a triterpenoid ancestry (Sangwan *et al.* 2008). Therefore, upstream in the biosynthetic-tree, they share a common route with
other triterpenoids which begins with isoprenogenensis followed by biosynthesis of oxidosqualene that serves as a common progenitor of a variety of triterpenoids (C_{30}) and sterols (mainly C_{28,29}). The follow up sequence of biosynthetic pathway for withanolide may be common with the ubiquitous sterols. The next phase of metabolic pathway may involve committed metabolic step(s) to generate parental pro-ergostanoid or ergostanoid intermediates which may be oxido-reductively processed to generate a myriad of withanolidal moieties. Finally, some of the withanolides appear to be subjected to terminal transformations like glycosylation, acylation etc.