1.1. Withania somnifera: an important medicinal plant

*Withania somnifera* (L.) Dunal is an important medicinal plant of the family Solanaceae. It is commonly referred to as Ashwagandha, Asgandh, Winter cherry or Indian ginseng and has been extensively used in Indian, Unani and African traditional medicines (Singh et al., 2015). It is also included in World Health Organization (WHO) monographs on selected medicinal plants (Mirjalili et al., 2009) and has been listed as top thirty-two selected priority medicinal plants by the National Medicinal Plant Board of India (www.nmpb.nic.in) due to its great demand in the domestic and international markets (Prajapati et al., 2003). In India, the plant is distributed in the north-western region of Himachal, Jammu and Punjab and it is commercially cultivated in Madhya Pradesh, Rajasthan, Andhra Pradesh and Uttar Pradesh (Kothari et al., 2003). Besides India, it occurs widely in the Middle East, Africa, Pakistan and the eastern Mediterranean region (Kumar et al., 2007, 2011; Patra et al., 2004). *W. somnifera* is mainly propagated through seeds and the plant grows well in red soil (slightly basic) receiving about 500-750 mm rainfall and the optimal temperature range is 20-32°C. The crop is harvested in about 180-210 days (Rajeswara Rao et al., 2012) and different parts of the plant are used for commercial purposes (Sharada et al., 2008; Yadav et al., 2010). It is cultivated in around 10,780 ha with a production of 8429 tones (Shrivastava and Sahu, 2013).

The plant is dicotyledonous, erect, small, woody evergreen, branched shrub that grows about two feet (30-150 cm) in height. It is minutely stellate-tomentose especially on the stem, leaf veins and calyx (Fig. 1.1). The leaves are simple petiolate, ovate, exstipulate, entire, acute and glabrous. Leaves on vegetative shoots are alternate and large while those on floral branches are opposite, arranged in pairs of one large and one small leaf. Axil contains a cymose cluster of 5 to 25 inconspicuous pale green flowers. Flowers are short-pedicellate 4-6 mm in diameter, gamosepalous, persistent, with acute linear lobes. In the fruiting stage the calyx becomes enlarged, inflated and completely encloses the fruit. Its corolla is gamopetalous, 5-lobed with lobes spreading or recurved, acute pubescent and greenish yellow while stamens arise from the base of petals and are slender filaments. Anthers are innate and oval whereas gynoecium is syncarpous, composed of minute swollen ovary subtended by a long slender style. Fruit is enclosed
in the green persistent calyx. It is green when unripe and turns orange-red when mature enclosing numerous small seeds (Singh and Kumar, 1998).

Fig. 1.1. *W. somnifera* – an important medicinal plant.

1.2. **Economic importance and chemical profile of *W. somnifera***


In traditional home medicine *W. somnifera*, leaves (Jayaprakasam *et al.*, 2003) and roots (Kumar *et al.*, 2011) are mainly used for herbal formulations. However, bark,
seeds (Kulmi and Tiwari, 2005) and fruits are used only rarely (Lal et al., 2006; Bolleddula et al., 2012). Recently, a total of 62 major and minor primary and secondary metabolites from leaves and 48 from roots have been identified, out of which 29 are common to both (Chatterjee et al., 2010) (Fig. 1.2). It is also reported that the distribution of secondary metabolites varies significantly with respect to different tissues, developmental stages and chemotypes (Chatterjee et al., 2010; Dhar et al., 2013). Withaferin A and withanine are major metabolites present in leaves, whereas withanolide A is a principle metabolite found in roots (Singh et al., 2015) (Fig. 1.2). Furthermore, NMR and HR-MAS NMR studies on different chemotypes of W. somnifera have shown a clear distinction in the metabolome of different organs (Namdeo et al., 2011; Bharti et al., 2011). NMR spectroscopy reveals that the leaves of W.somnifera have the widest array of metabolites which includes amino acids, flavonoids, lipids, sugars, organic acids, withanolides, trigonelline, ferulic acid, tryptamine and kaempferol glycosides (Namdeo et al., 2011). NMR spectra also reveals the presence of 2 types of withanolides: 4-OH and 5,6-epoxy withanolides (withaferin A-like steroids) and 5-OH and 6,7-epoxy withanolides (withanolides A-like steroids). It was further observed that ratio of these two withanolides was a major discriminating feature of W. somnifera leaf samples from different origins (Namdeo et al., 2011). Likewise, NMR methodology has also been employed for qualitative and quantitative analysis of metabolites in W. somnifera fruits in different chemotypes (Bhatia et al., 2013) and during different stages of development (Sidhu et al., 2011). The chemical profile also varies during different stages of fruit development. Relatively higher concentrations of withanolides, alanine, aspartate, choline, phosphocholine, sucrose and caffeic acid were recorded during early developmental stages whereas higher accumulation of citrate and withanamides was observed during maturation phase (Sidhu et al., 2011). Hence, for obtaining significant amount of desired bioactive compounds it is important to identify appropriate stage for harvesting (Sidhu et al., 2011).
1.3. **Morpho-chemotypic variations in *W. somnifera***

The genus *Withania* includes more than 23 species of which two species, *W. somnifera* and *W. coagulans* are found in India (Kumar *et al.*, 2011). Several reports document existence of considerable morphogenetic diversity in Indian populations (Kumar *et al.*, 2007). The variability was observed in plant height, number of branches per plant, number of seeds per berry, root length, root diameter and root yield. These morphological variations could be correlated with genetic variations. Genetic markers such as Random amplified polymorphic DNA (RAPD) and Amplified fragment length polymorphism (AFLP) have been employed to access genetic variability among different morpho-chemotypes of *W. somnifera* (Dhar *et al.*, 2006; Kumar *et al.*, 2007; Mir *et al.*, 2011). Study indicates the existence of two distinct clusters representing a clear distinction between cultivated and wild accessions (Mir *et al.*, 2011). Furthermore, this data to a large extent correlates with the morphological variations of different morpho-chemotypes. The genetic variability was also pronounced among the accessions collected from same geographical region (Dharmar and De Britto, 2011). In a comparative study, selectively amplified microsatellite polymorphic loci (SAMPL) assay and AFLP were used to assess the levels of genetic diversity in different *W. somnifera* genotypes (Negi *et al.*, 2006). Similar results were obtained employing inter simple sequence repeat (ISSR) markers suggesting a wide variation among morpho-chemotypes of *W. somnifera* (Bamhania *et al.*, 2013). Analysis of different morpho-chemotypes using AFLP and determination of their major active components suggests a correlation between the genetic and chemotypic variability (Dhar *et al.*, 2006). Apart
from molecular markers, isozymes, polypeptide polymorphism and withanolide content were also studied in selected accessions of *W. somnifera* in order to characterize them (Chaurasiya *et al.*, 2009).

Based on the chemical profile, *W. somnifera* plants have been classified into various chemotypes. Three different chemotypes of *W. somnifera* were recorded in Israel (Abraham *et al.*, 1968, 1975) and one each was recorded from South Africa and India (Kumar *et al.*, 2007). However, there is quite a possibility of occurrence of more than one chemotypes in India (Kumar *et al.*, 2007). The major steroidal lactones of chemotype I, II and III from Israel were withaferin A, withanolide D and withanolide E, respectively (Abraham *et al.*, 1968, 1975), whereas, Indian chemotype is rich in withanone and withaferin A (Anjaneyulu and Rao, 1997; Dhalla *et al.*, 1961; Kirson *et al.*, 1971). The main withanolides found in South African chemotype are withaferin A and withaferin D (Kirson *et al.*, 1970). Apart from these few high yielding varieties of *W. somnifera* have also been released. Jawahar Ashgand 20, a selection variety shows significantly higher root yield as compared to cultivated varieties (Nigam *et al.*, 1991). ‘Poshita’ and ‘Rakshita’ were released by CIMAP, Lucknow and have high root yield with improved chemical content (Misra *et al.*, 2001; Mathew *et al.*, 2005).

1.4. **Rationale of the thesis and objectives**

Recently there has been a global shift of preference towards natural chemicals and herbs for medicines and health supplements (Briskin, 2000). Herbal medicines are being widely accepted for their safety, efficacy, cultural acceptability, better compatibility with the human body and lesser side effects. WHO estimates that more than 80% of population in developing countries still relies primarily on traditional remedies such as herbs for their medicines (Haq, 2004). Approximately one quarter of prescribed drugs contain plant extracts or active ingredients obtained from plant source. Moreover, substitution of synthetic food antioxidants by natural antioxidants has gained significant momentum in last decade leading to screening of raw materials for identifying new antioxidants (Delgado-Adámez *et al.*, 2012). With such worldwide shift in consumer choice and preferences for herbal medicines, there have been intensive efforts towards propagation of medicinal plants using rational scientific approaches and understanding the bioactivities of compounds using a variety of model systems. *W. somnifera* has attracted interest of several workers all over the globe due to its diverse
pharmacological activities. However, its commercial cultivation is limited due to various factors such as long gestation period between planting and harvesting (Ray and Jha, 2001), narrow genetic base, self pollination (Kaul et al., 2005), low percentage of seed viability, germination (Vakeswaran and Krishnasamy, 2003) and seedling survival (Gupta et al., 1993; Nagraj and Reddy, 1985) and infestation by various pathogens (Sharma and Pati, 2012; Sharma et al., 2011). Moreover, due to diversity in chemical profile of *W. somnifera*, standardization of different herbal formulations is difficult. Extraction of bioactive compounds from natural sources is also limited as concentration of these compounds in the plant is generally low and the accumulation pattern of these compounds is highly susceptible to geographical and environmental conditions (Chatterjee et al., 2010). The concentration of withanolides usually ranges from 0.001 to 0.5% of dry weight in leaves and roots of the plant (Mirjalli et al., 2009). On the other hand, chemical synthesis of withanolides is also quite cumbersome owing to stereochemical ring closure and presence of chiral centers. Furthermore, synthetic production would yield only restricted quantities of a specific compound at high costs. Therefore, there is a need for the development of commercially viable alternatives for production of withanolides.

Substantial efforts have been made to understand the existing problems and formulate strategies for improvement of *W. somnifera*. *In vitro* propagation offers an attractive alternative that would lead to rapid multiplication of the elite genotypes, germplasm conservation and would also facilitate production of improved varieties when combined with modern tools of biotechnology. Developing an efficient micropropagation system would ensure steady supply of uniform plant material for the health sector. The potential of organ, tissue and cell culture system has been exploited for enhanced production of secondary metabolites of this immensely important medicinal plant. The plant has been subjected to tissue culture studies by several workers (Ghosh and Gupta, 2001; Furmanowa et al., 2001; Ray and Jha, 2001; Singh et al., 2006; Sivanesan 2007; Sabir et al., 2007; Fatima et al., 2011; Nayak et al., 2013; Gupta and Sahu, 2015). However, further improvement in existing protocols is much warranted. The review of literature suggests that the plant responds differentially to different culture conditions, plant growth regulators (PGRs) and treatments. Hence, it is imperative to study these factors in order to develop a reproducible and cost effective
micropropagation system. Furthermore, developing an efficient protocol for in vitro regeneration of *W. somnifera* is an essential prerequisite for genetic transformation studies (Pandey *et al*., 2010).

Numerous reports have shown existence of considerable genotypic and phenotypic variation among wild and cultivated species (Atal 1975; Misra *et al*., 1998; Dhar *et al*., 2006, 2008; Kumar *et al*., 2007). In some crosses involving different chemotypes, hybrid chemoprofiling has revealed a complex pattern of inheritance of various constituents (Kumar *et al*., 2007). Reports have also described morphometric and genetic diversity in correlation with withanolide markers (Misra *et al*., 1998; Dhar *et al*., 2006, 2008; Kumar *et al*., 2007). Different morpho-chemotypes provide an important resource for deciphering the biosynthetic pathway of withanolides. However, limited information of factors responsible for occurrence of morpho-chemotypic variations is major setback in its genetic improvement. This could be due to the limited efforts that have been made to characterize these morpho-chemotypes till date. However, in recent years biochemical and molecular studies have been undertaken to analyze the metabolic pathways required for the synthesis of withanolides (Madina *et al*., 2007; Senthil *et al*., 2010; Sharma *et al*., 2007; Patel *et al*., 2013). The molecular markers may be linked to genes of interest and selected phytochemicals allowing selection of the desired genotype and also for the identification of commercial varieties. Biogenesis of withanolides is complex process and sparse information is available on biosynthesis of withanolides and the relationships among different withanolides (Sabir *et al*., 2013). These metabolites are synthesized through the isoprenoid pathway, probably via both mevalonate and non-mevalonate pathways, wherein 24-methylene cholesterol is the first branching point towards the biosynthesis of different withanolides through a series of chemical reactions (Madina *et al*., 2007; Bhat *et al*., 2012). Therefore, identification of pharmaceutically important secondary metabolites and their experimental validation as well as understanding their biosynthesis, transport, accumulation, and modulation is much warranted (Singh *et al*., 2015).

Keeping the above in mind, the present study was designed with the following objectives:
1. Development of an efficient *in vitro* propagation system in *W. somnifera*.

2. Characterization of different morpho-chemotypes of *W. somnifera*.

3. Characterization of key genes of withanolide biosynthesis and transport in *W. somnifera*.