Introduction
INTRODUCTION

Biodiversity encompasses all biological entities occurring as an interacting system in a habitat or ecosystem and plants constitute a very important segment of such biological systems. Biodiversity of plants, collectively known as ‘plant genetic resources’, is a key component of any agricultural production system—indeed, of any ecosystem, without which, natural evolutionary adjustment of the system to the changing environment and biotic conditions would be impossible. Plant diversity is an irreplaceable resource, providing raw materials for introduction, domestication as well as improvement programmes in agriculture and forestry (Manzanera et al. 1996; Ramakrishnappa 2003)

India is a treasure chest of biodiversity which hosts a large variety of plants and has been identified as one of the eight important ‘Vavilorian’ centres of origin and crop diversity. Although its total land area is only 2.4% of the total geographical area of the world, the country accounts for 8% of the total global biodiversity with an estimated 49,000 species of plants of which 4,900 are endemic (Kumar and Asija 2000). India is one of the world’s top 12 mega diversity countries with 10 biogeographic regions and has over 40 sites which are known for their high endemism and genetic diversity (Singh and Chouwdhery 2002).

Among ancient civilization, India has been known for the richest arrays of registered and relatively well known repository centre harbouring a multitude of medicinal plants (Raven 1998). Medicinal plants which constitute a segment of the flora provide raw material for the use in all the indigenous systems of medicine in India, where about 1800 species are used in classical Indian systems of medicines, Ayurveda uses 1200, Siddha utilizes 900, Unani 700, Amchi 600 and 450 species are used by Tibetan. The age old traditional values attached with the various forest types and the varieties of forest products (medicinal plants) have gained tremendous importance in the present century (Stein 2004). According to the World Health Organization (WHO), 80% of the population of developing countries relies on traditional medicines, mostly in the form of plant drugs for their health care need (Vines 2004). Additionally, modern medicine contain plant derivatives to
the extent of about 25% on account of the fact that the derivatives of medicinal plants are non-narcotic having no side effects, the demand of these plants is on the increase in both developed and developing countries. In recent years, the growing demand for herbal products has led to a quantum jump in volume of plant material traded within and outside the country. Over 1.5 m practitioners of Indian system of medicine in the oral and codified streams use medicinal plants in preventive, promotional and curative applications. It is estimated that there are over 7800 medicinal drug manufacturing units in India, which consumes about 2000 tonnes of herbs annually (Singh 2001). The annual export of medicinal plants from India is valued at Rs. 1200 million.

Over 70% of the plant collection involves destructive harvesting because of the use of the parts like roots (29.6%), barks (13.5%), wood (2.8%), rhizome (4%), stem and the whole plant in case of herbs. An estimate of the EXIM bank projects international market of medicinal plants related trade over US $ 60 billion per year that is growing at the rate of 7% per year. The global ayurvedic products market is reportedly worth $ 14.2 billion (Indian Express P-11, May 29, 2005). Planning Commission Task Force has targeted exports of herbal products of Rs. 3000 crore by the year 2005 and Rs. 10,000 crore by the year 2010 (Tiwari 2000). World Health Organization (WHO) has estimated the growing demand for medicinal plant based raw materials at the rate of 15 to 25% annually, and likely to increase more than US $ 5 trillion in 2050 (Kiran et al. 2009).

The vast majority of the people on this planet still rely on their traditional material medica (medicinal plants and other materials) for their everyday health care needs. Medicinal plants typically contain mixtures of different chemical compounds that may act individually, additively or in synergy to improve health. “For every disease that arises on this planet, plants or herbs give cure” (Singh and Rao 2000; Hedge et al. 2007).

Phytodiversity is increasingly threatened at ecosystems, species and genetic level. The rapid destruction of world’s diverse ecosystems, especially in tropics has led to conclude that a quarter of the earth’s total biodiversity is in serious risk of
extinction during the next twenty- thirty year. The genetic diversity of medicinal plants in the world is getting endangered at an alarming rate because of ruinous harvesting practices and over-harvesting for the production of medicines, with little or no regard to the future. This factor poses a serious threat to the genetic stock and the biodiversity. During the period of 1996-2004, a total of 8321 plant species were added to the International Union for the Conservation of Nature and Natural Resources (IUCN) Red list of threatened species (IUCN 2004).

It would be essential to adopt a holistic interdisciplinary approach, have a scientific basis of the understanding of the plant systems, new innovations and their conservation for utilization in future on a sustainable basis. According to World Conservation Strategy (IUCN, UNEP and WWF 1980); Conservation is defined as “the management of human use of the biodiversity so that it may yield the greatest sustainable benefit to the present generation while maintaining its potential to meet the needs and aspirates of future generations” (Udvardy 1984). Conservation and use of genetic diversity for sustainable ecosystem or agro-ecosystem should be continuous to meet food, clothing, shelter and health requirements of India’s growing population. To safeguard the existing bioresources for future generations and to achieve sustainable development based on the use of available genetic resources, conservation of phytodiversity is of immense importance. Therefore, *in situ* and *ex situ* management programmes are necessary approaches for its conservation and consequently plant improvement. *In situ* conservation involves maintaining genetic resources in their natural habitats/wild whereas, *ex situ* conservation involves conservation outside the native habitat and is generally used to safeguard populations in danger of destruction, replacement or deterioration.

Advances in molecular and cell biology have led to the development of a whole range of techniques for manipulating genomes, collectively named “Biotechnology”. The biotechnological derived solutions for low productivity, biotic and abiotic stresses as well as improved nutritional or medicinal content built into the genotype of plants could reduce use of agrochemicals and water, thus
promoting sustainable yields, food security and added value integration. In recent years, plant biotechnology has made an impressive progress as one of the frontiers of biotechnology of scientific and economic importance. To cope up with the present alarming situation, the recent exciting development in plant biotechnology have come as a boon and has been regarded as a priority area for technology transfer, because genetically modified food, feed and fibers are vital concern to the developed world (Ives and Bedford 1998; Altman 1999; Tripathi and Tripathi 2003; Canter et al. 2005).

The plant biotechnology has provided a large number of tools and techniques which are more efficient in generating novel genetic variability and making selection procedure, more precise and reproducible. There are four main areas of biotechnology which can directly assist plant conservation programmes:

A. Molecular marker technology
B. Molecular diagnostic
C. Tissue culture (in vitro technologies)
D. Cryopreservation

In vitro culture-a key tool of plant biotechnology which exploits the totipotency nature of plant cells, a concept proposed by Haberlandt (1902), is the science of growing plant cells, tissues or organs isolated from the mother plant on artificial media under aseptic conditions. The powerful techniques in plant cell and tissue culture coupled with most sophisticated and analytical tools, have offered mankind the great potency of exploiting the totipotent biosynthetic and biotransformation capabilities of plant cells under in vitro conditions (Stockigt et al. 1985). Plant cell, tissue and organ culture techniques have emerged as escapable biotechnological tool with the possibilities of complimenting and supplementing the conventional method in plant breeding, plant improvement, biosynthetic pathways, etc. It plays a major role in conservation of germplasm, rapid clonal propagation and regeneration of genetically manipulated superior clones, production of secondary metabolites and ex vitro conservation of valuable
phytot diversity. The various applications of plant tissue culture in different fields have been presented in figure 1.

Plant tissue culture has been viewed as a key technology for enhancing the capabilities for the production of large quantities of planting material of selected elite, high yielding varieties so as to boost production and productivity and also to conserve the fast diminishing species. This technology owns a unique distinction as the quick and easy method of deriving plants with identical genetic constitution (Hussey 1986). The technique provides an easy method to study the mechanism of cell differences, thereby providing an experimental approach to link the genotype with phenotype. It has emerged as an invaluable aid which has a tremendous potential in solving global problems.

Conventional propagation is time consuming and labour intensive and requires availability of a large number of plants for plantation or aorestation which has become a challenging task (Ray et al. 2005). In vitro plants may also be used continuously in reforestation programmes (Bionda et al. 2007; Anis et al 2009; 2011 and 2012). The uses of in vitro germplasm storage in plant biotechnology programmes has a growing significance, as it improves the efficiency of research activities and secure the valuable products of such activities for both scientific and commercial purposes (Lynch 1999).

In vitro method also provides a supplemental approach for addressing target 8 of the global strategy for Plant Conservation (Wyse Jackson 2004), when traditional methods of ex-situ conservation are not adequate. On account of urbanization and environmental pollution, the loss of forest cover, land degradation and depletion of rich valuable genetic resources has accelerated considerably with time.

The recent development in clonal micropropagation of plants has been of great help in the cultivation of medicinal plants by providing standard quality planting material (George and Sherington 1984) and widely used for the commercial propagation and re-vegetation of large number of plant species, including various medicinal plants (Rout et al. 2000; Siddique and Anis 2009a
Fig 1. Applications of plant tissue culture (Source: www.ePlantScience.com)
and b; Anis et al. 2009; 2010; Naz et al. 2011; Perveen et al. 2011; Jahan et al. 2011a and b; Fatima et al. 2011a; Fatima and Anis 2012a). Many medicinal and aromatic plants have been successfully used for in vitro regeneration and mass multiplication through the use of various explants (Casado et al. 2002; Arya et al. 2003; Nalawade and Tsay 2004; Faisal et al. 2005; Chaturvedi et al. 2007; Karuppusamy et al. 2009; Sharan et al. 2010; Siddique et al. 2010; Anis et al. 2010; 2011; 2012; Fatima and Anis 2011b; Varshney and Anis 2012).

In the present investigation, an important medicinal plant species namely, 

**Withania somnifera** L. (Dunal.) has been selected for its large scale propagation which can be used for re-introduction and conservation purposes through various biotechnological approaches.

1. **Withania somnifera** L. (Dunal)

1.1. **Scientific classification**

<table>
<thead>
<tr>
<th>Synonym</th>
<th>Physalis somnifera L., Physalis flexuosa L.</th>
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<tbody>
<tr>
<td>English name</td>
<td>Winter cherry</td>
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<tr>
<td>Hindi name</td>
<td>Asgandh</td>
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<tr>
<td>Common name</td>
<td>Ashwagandha</td>
</tr>
<tr>
<td>Family</td>
<td>Solanaceae</td>
</tr>
<tr>
<td>Plant parts used</td>
<td>Leaves, root and seeds</td>
</tr>
<tr>
<td>Propagation</td>
<td>By seeds</td>
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</tbody>
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1.2. **Distribution**

W. somnifera or Indian ginseng is a highly reputed plant of Indian system of medicine consist of about 90 genera and over 200 species distributed in the tropical and temperate regions but chief centre of their distribution is Central and South America. The plant is widely distributed in the drier parts of India (more than 4000 ha) upto an altitude of 2000 m in Himalayas (Anonymous 1976).

1.3. **Botanical description**

W. somnifera is an erect greyish, stellate tomentose under shrub (30-75 cm high) with long tuberous roots. Leaves are alternate or sub-opposite, broadly ovate to oblong, petiolate, sub-acute and entire with lamina. Flowers are gamosepalous,
accrescent and inflated in a fruit. The corolla is campanulate, greenish yellow. The ovary is ovoid or globose, glabrous and many ovuled. The style is filiform and stigma is 2 lobed. Fruit is globose berry, orange red. Seeds are many discoid, yellow and reniform. The chromosome number is 2n=48 (Anonymous 1976).

1.4. Chemical constituents
At present more than 12 alkaloids, 40 withanolides and several sitoindosides (a withanolide containing a glucose molecule at carbon 27) have been isolated and reported from aerial parts, roots and berries of *Withania* species. The major chemical constituent, withanolides are mainly localized in leaves and their concentration usually ranges from 0.001 to 0.5 % dry weight (DW). The leaves contain withanolides like withaferine A (Devi 1996) and contain foreign organic matter (72 %) acid insol, ash (1.2 %), alcohol solution matter (16 %). The several other alkaloids present vary between 0.13-0.31 %. The roots also contain starch, reducing sugar, glucose, dulcitol, withaniol (0.08 %). The free amino acids include aspartic acids, glycine, tyrosine, alanine, proline, tryptophan, glutamic acid and cystine which also too have been identified in the roots. Other steroidal compounds isolated are non lactonic ergostane derivatives (Sitoindosine-VIII), besides β-sitosterol, and β- sitosterol glucoside (Bhattacharya et al. 1987). Several alkaloids which occur to the extent of 0.3 % have been characterized and identified as nicotine, anajerine, anhygrine, isopelletierine, and withasomnine. Recently, a quantification of withanolides D and withaferine A was developed using HPLC (Ganzera et al. 2003).

1.5. Medicinal properties and uses
In Ayurveda, *Withania* is widely claimed to possess potent, aphrodisiac, rejuvenative and life prolonging properties. It is also used as a general energy-enhancing tonic known as Medharasayana, used to promote learning and a good memory, and in gastritis problem (Williamson 2002). The plant was traditionally used to promote youthful vigour, endurance, strength and health, nurturing the time elements of body and increasing the production of vital fluids, muscle fat, blood, lymph, semen and cells. It also helps counteract chronic fatigue, weakness,
dehydration, bone weakness, loose teeth, thirst, impotency, premature ageing, emaciation, debility and muscle tension. Bruised leaves and fruits are locally applied to tumour and tubercular glands, carbuncles and ulcers (Kapoor 2001). The leaves contain withanolides like withaferine A that exhibit anti-tumor and antibacterial properties (Kurup 1956; Devi and Sharada 1992; Devi 1996). The roots are also used in constipation, senile debility, rheumatism, general debility, nervous exhaustion, loss of memory, loss of muscular energy and spermatorrhoea (Singh and Kumar 1998). In Ayurveda, the roots are also prescribed for gynaec disorders, bronchitis, arthritis, rheumatism, inflammation, fever and skin diseases etc.

1.6. Conventional propagation and its limitations

Ashwagandha propagates through seeds but a reduced span of viability and also low germination rate restricts its propagation through seeds even after stratification (Dewir et al. 2010). Moreover, the genetic diversity of the species in India is now getting endangered (Antonisamy and Manikam 1999) at an alarming rate, because of lack of proper cultivation, ruinous harvesting practices for the production of medicines, loss of habitats and the illegal, indiscriminate collection of this plant material from its natural habitat pose a serious threat to its existence in the wild.

Direct regeneration or regenerant differentiation of whole plant is essential for plant improvement through biotechnological interventions. Therefore, development of a practicable, efficient and rapid technique for the propagation and mass multiplication of *W. somnifera* has become imperative in order to reduce the existing pressure on natural populations and to provide a constant supply of plant materials for the pharmaceutical industries round the year, irrespective of seasonal constraints. The wild nature of the plant is the other necessity of micropropagation and to bring it in cultivation practices for the production of improved quality and medicinal values for future use.

Owing to the immense importance of the plant many authors have reported regeneration in *W. somnifera* using various explants (Kulkarni et al. 2000; 2006; Sivanesan 2007; Sivanesan and Murugesan 2008; Ghimire et al 2010, Logesh et al.
2010; Kumar et al. 2011), but none of the reports provide a comprehensive and detail study on its regeneration systems which could apprehend the problems faced by the plant.

Thus, considering the immense possibilities offered by the application of tissue culture techniques and current status of W. somnifera, present study has been attempted with an approach to standardize a reproducible regeneration protocol in vitro, followed by successful ex vitro establishment of regenerated plants.

1.7. Objectives

The present experimental work was undertaken during the study with the following objectives (Fig. 2);

1. To establish and proliferate the aseptic cultures from in vitro raised juvenile explants.

2. To improve the regeneration protocol with the manipulation of culture conditions for obtaining maximum multiplication and regenerant differentiation in somatic tissues from various explants.

3. To optimize the mineral nutrient composition for maximum regeneration and subsequent proliferation.

4. To optimize the parameter for synthetic seed production and to study their conversion potential under in vitro conditions

5. To standardize the technique for rooting in regenerated microshoots.

6. To carry out the histological examination of regenerated shoot buds.

7. To standardize the hardening and acclimatization procedure in culture.

8. To study the various physiological and biochemical parameters during ex vitro establishment of in vitro raised plantlets.

9. To determine the genetic fidelity of in vitro raised field established plantlets using RAPD/ISSR markers.
Explanation of Fig. 3: Diagrammatic representation of work plan