INTRODUCTION
Chapter 1 INTRODUCTION

1.1. Global and national scenario

Planet earth is endowed with rich variety of life forms and the teaming millions of these living organisms have been well-knit by the laws of nature. The inter-dependence of various life forms starting from the unicellular primary producers to the complexly built higher plants and animals is unique feature of this green planet. Biodiversity, as this assemblage of life forms is referred to, has now been acknowledged as the foundation for sustainable livelihood and food security. Also, scientists have estimated that more than 50 million species of plants and animals including invertebrates and microorganisms occur on earth and hardly 2 million of them have been described by man so far.

Forests are one of the most valuable eco-systems in the world, containing over 60% of the world's biodiversity. This biodiversity has multiple social and economic values, apart from its intrinsic value, varying from the important ecological functions of forests in terms of soil and watershed protection to the economic value of the numerous products which can be extracted from the forest. For many indigenous and other forest-dependent people, forests are their livelihood. They provide them with edible and medicinal plants, bushmeat, fruits, honey, shelter, firewood and many other goods, as well as with cultural and spiritual values.

On a global scale, all forests play a crucial role in climate regulation and constitute one of the major carbon sinks on earth, their survival thus preventing an increase in the greenhouse effect. Forest trees are recognized as a raw-material base for industrial and domestic wood products, which provides perpetually renewable energy, fiber and timber. The economic benefits of planted forests have led to their widespread adoption throughout
the world. Globally, 48% of the forest plantation is established for industrial use, 26% for non-industrial use (fuelwood, soil and water conservation) and the remaining 26% is not specified. The current extent of world’s plantation forest area is about 187 million hectares (m ha) with the annual planting of 4.5 m ha (FRA 2001).

India, on the tropic of cancer, is a known mega biodiversity center with 8% of the global biodiversity in 2.4% land. The country is 10th among plant rich countries of the world and 4th among the countries of Asia. It poses out 20,000 species of higher plants, one third of it being endemic and 500 species are categorized to have medicinal value (Krishnan et al. 2011). In addition, it is one of the largest hardwood plantation resources comprising about 32.5 m ha with *Eucalyptus*, *Acacia* and Teak as major species (FRA 2001) whereas, Bamboo species occupy 8.96 m ha. The annual planting target of India is about 3 m ha, which requires roughly 6100 m seedlings for 10 major species, viz. *Eucalyptus*, Bamboos, *Acacia*, *Albizia*, *Prosopis cineraria*, *P. juliflora*, *Casuarina*, *Dalbergia*, Conifers and Teak (Gurumurthi 1994).

The National Forestry Action Programme (NFAP) and National Forest Policy of India have identified the expansion of area under forest plantations with increased productivity as one of the important thrust areas in forestry. Significance of plantation establishment and the use of improved planting stock have also been emphasized in a number of recent reviews (Kanonshi 1997; Evans 1998; Pandey and Ball 1998; Yasodha et al. 2004).

Besides other valuable products several trees are recognized for their medicinal and pharmaceutical importance. Today, at a local level, an extremely wide range of plant species is used for medicinal purposes, the WHO has listed over 21,000 plant names (including synonyms) that have reported medicinal uses around the world. Thousand of medicinal and aromatic plants are already been reported in Indian literature pertaining to
medicinal importance. In this context, India has recognized just over 7,500 species having true medicinal value, but more than 500 traditional communities use about 800 plant species for curing different diseases (Kamboj 2000).

In developing countries as a whole, it is estimated that over 6,000 plants are utilized in traditional medicines (Srivastava et al. 1995). In India, about 33% plant species are trees while about 52% are shrubs and herbs. About 1500 plant species are used for ethical and classical formulations and home remedies based on Indian System of Medicines (ISM) such as Ayurveda, Siddha and Unani. It is estimated that Indian consumption alone of these medicinal plants (188 tonnes) is used for culinary purposes and about 12 tonnes are consumed for medicinal and cosmetic preparations. In the last century, roughly 121 pharmaceutical products were formulated based on the traditional knowledge obtained from various sources. Currently, 80% of the world population depends on plant-derived medicine for the first line of primary health care for human alleviation because it has no side effects (Vines 2004).

Worldwide, many plant species are threatened with extinction because of the gradual disappearance of the terrestrial natural ecosystem caused by the various human activities. Often, this is due to the clearing of indigenous vegetation for agriculture and the resulting erosion, salinization and invasion of alien species, but more recently climate change is looming as a significant new threat. More than 50% of the world plant species are endemic to 34 global biodiversity hotspots which once covered 15.7% of the earth’s land surface and which are now reduced to 2.3%. The International Union for Conservation of Nature Red List of Threatened Plants, first published in 1998 (IUCN 1998), lists more than 8,000 species currently in danger (IUCN 2010). There are numerous estimates of predicted extinction rates but the consensus view is that 15-20% of all plant species could become extinct by 2020.
Besides this, demographic changes, the growing size of population of the world and increasing urbanization have had and will continue to have, major impact on forest cover and condition, demand for wood and non-wood forest products and the ability of forests to fulfill essential environmental functions. Political and economic trends affecting the forestry sector are decentralization, privatization, trade liberalization and globalization of world economy, and overall economic growth coupled with a widening gap between rich and poor in many countries.

Deforestation and forest degradation are occurring in dry lands and uplands areas. These already have limited forest cover and are fragile environments susceptible to soil erosion and other forms of degradation. In these areas, the communities are highly dependent on forests for food, fuel and income. The most important direct causes of deforestation include logging, the conversion of forested lands for agriculture and cattle-raising, urbanization, mining and oil exploitation, acid rain and fire. However, there has been a tendency of highlighting small-scale migratory farmers or "poverty" as the major cause of forest loss. Such farmers tend to settle along roads through the forest, to clear a patch of land and to use it for growing subsistence or cash crops. In tropical forests, such practices tend to lead to rapid soil degradation as most soils are too poor to sustain agriculture. Consequently, the farmer is forced to clear another patch of forest after a few years. The degraded agricultural land is often used for a few years more for cattle grazing. This is a death sentence for the soil, as cattle remove the last scarce traces of fertility. The result is an entirely degraded piece of land which will be unable to recover its original biomass for many years.

To maintain and sustain forest vegetation, conventional approaches have been exploited for propagation and improvement, but tree breeding efforts are restricted to the most valuable and fast growing species. The major challenge for the tree breeder is to quickly transfer the ever-improving material from the breeding programmes into plantations. The basic method of
achieving such transfers is the use of seeds from orchards or clonal propagation. However, such methods are limited with several inherent bottlenecks because trees are generally slow growing, long lived, sexually self-incompatible and highly heterozygous plants. Due to the prevalence of high heterozygosity in these species, a number of recessive deleterious alleles are retained within populations, resulting in high genetic load and inbreeding methods such as selfing and backcrossing, and makes it difficult to fix desirable alleles in a particular genetic background (Williams and Savolainen 1996). Thus, conventional breeding is rather slow and less productive and cannot be used efficiently for the genetic improvement of trees.

Tissue culture and other biotechnological approaches offer tremendous scope towards the desired objectives. Exploration, collection, characterization, evaluation, domestication/cultivation and ex situ conservation in gene banks eventually support their sustained use supplying quality planting material and certified raw drugs.

1.2. In vitro approaches
The worldwide importance of forestry, summed to the lengthy generation cycles of tree species, makes unavoidable the development of new technologies that complement conventional tree breeding programs in order to obtain improved genotypes. In this regard, a new set of tools has become available in the past twenty years that combined with traditional plant breeding will allow people to generate products, the genetically improved varieties of the future. This set of tools, comes under the general title of ‘Biotechnology’. Three specific biotechnological tools have been successfully used in several programs of plant conservation, namely, (1) tissue culture techniques for in vitro propagation, (2) the use of molecular marker to assess the degree of variability among population, and (3) techniques of long-term conservation such as encapsulation and cryopreservation.
Plant tissue culture techniques are particularly relevant, becomes an alternative not only for large scale propagation of individuals that are threatened, reduce production costs and increase gains to the industry, but also to provide ecological advantages as in phytoremediation or in the establishment of artificial plantings in weed-infested site. The use of more efficient trees may reduce the soil areas required to produce the goods and services derived from tree plantations, thus reducing the use of natural ecosystems for sustainable and agricultural purposes. In India, the major accomplishment has been made in the in vitro propagation of various plant species, including forest trees like teak, eucalypts, bamboos, sandal, Vitex negundo, Albizia lebbeck, Tecomella undulata (Sharma 2002; Anis et al. 2011).

1.3. Constraints with tree tissue culture
Tissue culture of tree species often poses problems which are either absent or of lesser significance when culturing herbaceous species. Tree species have been cultured in vitro since 1930’s. Since that time much progress has been made in the culture of tissues, organs, cells and protoplasts of tree species. That is not to say that all woody plants, including forest trees, can be induced to grow and differentiate in vitro. Some do, while others are still recalcitrant. In general, juvenile tissues from trees are more responsive to in vitro manipulations than the mature tissues. Trees have long generation cycles and have an extended vegetative phase, ranging from 10 to 50 years. During the juvenile phase, starting from the embryo and perhaps lasting upto a decade, tissues from tree species are responsive to in vitro conditions. As maturation sets in, tissues from mature trees become less responsive in tissue culture. Other than age of a tree, the response of an explants/ tissue is also determined by the genotype, physiological state of the tissue, time of the year when the explant is cultured, and the composition of medium. Also, dependence on the mature or field-grown materials poses surface sterilization problems (Pierik 1997).
Even with these limitations, tissue culture has been successfully applied to a wide range of trees and its implication has been mentioned in some exhaustive review of Thorpe and Harry (1991), Harry and Thorpe (1994), McCown (2000), Giri et al. (2004), Yasodha et al. (2004) and Anis et al. (2011).

1.4. Plant tissue culture for trees
Since the beginning of domestication and cultivation of plants, human beings are looking for techniques that help him to produce maximum number of individuals from the minimum number or quantity of propagules. Plant tissue culture is the ultimate finding of his enquiry towards mass multiplication of plants using minimum quantity of propagules. Tissue culture refers to the aseptic growth of cells, tissues or organs in artificial media. Tissue culture approaches to conservation are appealing as the procedure ensures propagation is possible for species where:
1. Source explants are in limited abundance.
2. Inbreeding has resulted in lowered seed yields (Hendrix and Kyhl 2000).
3. Species possess complex, often unresolved seed dormancy mechanisms (Merritt and Dixon 2003; Merritt et al. 2007).
4. Seed availability is hampered by low and erratic seed set and viability due to environmental stresses such as drought, predation or disease (or simply a paucity of information on optimal time for seed collection).

Some of the advantages of this technique are heterozygous materials may be perpetuated without much alteration, easier, faster, dormancy problem eliminated and juvenile stage reduced. It is also a mean for perpetuating clones that do not produce viable seeds or that do not produce seeds at all. It is now a well established technology. Like many other technologies, it has gone through different stages of evolution; scientific curiosity, research tool, novel applications and mass exploitation.
Today plant tissue culture applications encompass much more than clonal propagation and micropropagation. The range of routine technologies has expanded to include somatic embryogenesis, somatic hybridization, virus elimination as well as the application of bioreactors to mass propagation. These techniques, particularly micropropagation methods, are currently in use for the large scale multiplication of important tree species, woody biomass production, production of valuable secondary metabolites including pharmaceuticals, pigments and other chemicals and conservation of elite and rare germplasms (Giri et al. 2004).

In general three modes of in vitro plant regeneration have been in practice, organogenesis, embryogenesis and axillary proliferation. The difference mainly matters when it relates to the genetic stability of the resulting micropropagated plants; the obvious option then would be axillary and adventitious shoot proliferation. In vitro micropropagation has proved in the recent past a means for supply of planting material for forestry (Ahuja 1993; Lakshmisita and Raghavaswamy 1998).

In the present investigation, an important forest tree species namely, Balanites aegyptiaca (Del.) L. has been selected for its large scale propagation which can be used for reintroduction and conservation purposes through biotechnological approaches.
1.5. Taxonomy

1.5.1. Scientific classification

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Plantae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Division</td>
<td>Magnoliophyta</td>
</tr>
<tr>
<td>Class</td>
<td>Magnoliopsida</td>
</tr>
<tr>
<td>Order</td>
<td>Zygophyllales</td>
</tr>
<tr>
<td>Family</td>
<td>Zygophyllaceae/Balanitaceae</td>
</tr>
<tr>
<td>Genus</td>
<td>Balanites</td>
</tr>
<tr>
<td>Species</td>
<td>aegyptiaca</td>
</tr>
</tbody>
</table>

1.5.2. Binomial name: *Balanites aegyptiaca* (Del.) L.

1.5.3. Common names: In English it is called Desert date, Jericho balsam, Egyptian Balsam, Balanos, Zacum oil plant, Soapberry tree; in Arabic it is known as Lalob, Hidjihi, and Heglig. In Hindi it is called Hingota.

1.5.4. Habitat

It is indigenous to all dry lands south of Sahara and extending southwards (Hall and Walker 1991; Sidiyene 1996; Sands 2001). It is found in Tropical and Northern Africa, Syria, West Asia, Sudan, Egypt, neighbouring parts of East and West Africa particularly Senegal, Nigeria, Arabia and Burma. In India, it is distributed in the drier parts of Western Rajasthan and from South-East Punjab to West Bengal and Sikkim (Amalraj and Shankarnarayan 1998; Anonymous 2001). It can be found in many kinds of habitat, tolerating a wide variety of soil types, from sand to heavy clay, and climatic moisture levels, from arid to subhumid. It is relatively tolerant of flooding, livestock activity and wildfire (Von Maydell 1984).
1.5.5. Morphological description

It is multibranched, spiny shrub or tree up to 10 m tall. Crown is spherical, in one or several distinct masses. Trunk is short and often branching from near the base. Branches are pubescent; with axillary spreading spines up to 4 cm. Bark is dark brown to grey, deeply fissured. Branches are armed with stout yellow or green thorns up to 8 cm long. Leaves grow with two separate leaflets; leaflets are obovate, asymmetric, 2.5-6 cm long, bright green, leathery, with fine hairs when young. Flowers grow in fascicles in the leaf axils and are fragrant, yellowish green. They are small, inconspicuous, hermaphroditic and pollinated by insects. Fruit is a rather long, narrow drupe, 2.5-7 cm long, 1.5-4 cm in diameter. Young fruits are green and tomentose, turning yellow and glabrous when mature. The pulp is bitter-sweet and edible. The seed, a pyrene stone, is 1.5-3 cm long; light brown, fibrous and extremely hard. It makes up 50-60% of the fruit. There are 500-1500 dry, clean seeds per kg (Anonymous 2001).

1.5.6. Active constituents

Phytochemical investigations on *Balanites aegyptiaca* yielded in the isolation of several classes of secondary metabolites, many of which expressed biological activities such as cumarins, flavonoids and steroidal saponins (Sarkar et al. 2000). Saponins (Diosgenin) are the major compounds which have been found in the various parts of the plant extracts (Pettit et al. 1991; Farid et al. 2002; Lindin 2002; Staerk et al. 2006).

Two furostanol glycosides and 6-methyl-diosgenin were obtained from the fruits (Kamel 1998). The fruit pulp also contains five steroidal saponins, designated as balanitisin A, B, C, D and E. The kernel contain balanitisin F and G. Chemically, balanitisin A has been identified as Diosgenin-3-O-α-D-glucopyranosyl (1-3)-O-α-D-glucopyranosyl- (1-4)-β-L-rhamnopyranoside. The diosgenin content of the fruits varies from 0.3-3.8% (Anonymous 2001). The kernel is very rich in both oil (46.0-55.0%) and protein (26.0-34.0%)
(Mohamed et al. 2002). The defatted seeds are a source of diosgenin, so the fruit wall which contains 1.5% diosgenin on fresh weight basis.

The roots and bark of the plant also have several steroidal saponins, yamogenin glycosides, were isolated (Pettit et al. 1991). The root wood contains balanitisin H and the stem wood balanitisin I. More recently five new steroidal glycosides were isolated from the roots of the plant (Farid et al. 2002). The diosgenin content of the roots varies from 0.3-1.5%. The leaves contain a saponin of diosgenin, stigmasterol and a small amount of free diosgenin (Anonymous 2001).

1.5.7. Medicinal properties and uses
Various parts of the *Balanites aegyptiaca* have been used for folk medicines in many regions of the Africa and Asia (Hall and Walker 1991; Neuwinger 1996; Mohamed et al. 2002). Literature has revealed antifeedant, antidiabetic, molluscide, antihelmintic, anti-inflammatory, antimicrobial, analgesic and contraceptive activities in various plant extracts (Liu and Nakanshi 1982; Kamel et al. 1991; Ibrahim 1992; Rao et al. 1997; Gaur et al. 2009).

The bark, unripe fruits and leaves of this plant are reported to have antifertility, purgative and antidysentric properties. The kernel oil possesses anti-bacterial and anti-fungal properties and is reported to be used in the treatment of skin diseases, burns, excoriations and freckles. The fruit and seed is considered useful in whooping cough and colic. The powdered seed is used for easy childbirth and decoction of root is reported to be emetic (Anonymous 2001).

Recently, the study has endeavored to utilize esters of *Balanites aegyptiaca* as a fuel for diesel engine. Ester developed from balanites oil by the transesterification process has been investigated for its properties and the
engine performance (Deshmukh and Bhuyar 2009). Also, it has been used as a model for the utilization of bioresources in the Israeli Arava desert and potentially other similar areas for cost-effective biodiesel production (Chapagain et al. 2009).

1.5.8. Other uses
Many parts of the plant are used as famine foods in Africa; the leaves are eaten raw or cooked, the oily seed is boiled to make it less bitter and eaten mixed with sorghum, and the flowers can be eaten. The tree is considered valuable in arid regions because it produces fruit even in dry times. The fruit can be fermented for alcoholic beverages. The oil is used as cooking oil. The seed cake remaining after the oil is extracted is commonly used as animal fodder in Africa. The tree is managed through agroforestry. It is planted along irrigation canals and it is used to attract insects for trapping. The wood is used to make furniture and durable items such as tools, and it is a low-smoke firewood and good charcoal. The smaller trees and branches are used as living or cut fences because they are resilient and thorny. The tree fixes nitrogen. The bark yields fibers, the natural gums from the branches are used as glue, and the seeds have been used to make jewelry and beads.

1.5.9. Conventional propagation method and its limitations:
Conventionally, *Balanites* is propagated by seeds and through root suckers. These methods are not very successful being season-dependent, space requiring and cumbersome in nature. Another bottleneck is the propagation through seeds or root sucker is also not very efficient in producing sufficient number of planting stocks due to poor germination of seeds, and root sucker production is strictly age dependent (Siddique and Anis 2009a). Therefore, we need to sort to alternate methods like micropropagation for rapid multiplication of planting stock material.
Tissue culture studies in *Balanites aegyptiaca* Del. till date

Although the plant is having huge manifold applications for mankind, there are only a couple of reports available on micropropagation for its conservation and replenishment as shown in the table below.

<table>
<thead>
<tr>
<th>Mode of regeneration</th>
<th>Explant used</th>
<th>Response</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct</td>
<td>Root</td>
<td>Adventitious shoots, plantlet regeneration</td>
<td>Gour et al. (2005)</td>
</tr>
<tr>
<td>Indirect</td>
<td>Cotyledon</td>
<td>Callus, Multiple shoot formation, plantlet regeneration</td>
<td>Gour et al. (2007a and b)</td>
</tr>
<tr>
<td>Direct</td>
<td>Node</td>
<td>Axillary multiple shoot formation, plantlet regeneration</td>
<td>Ndoye et al. (2003); Siddique and Anis (2009a); Anis et al. (2010)</td>
</tr>
</tbody>
</table>

1.5.10. Objectives undertaken during the study

After reviewing the existing status of *Balanites aegyptiaca* Del., and the published protocols on its *in vitro* propagation where less number of shoots has been obtained, represented the need of more formal research effort on the subject. Therefore, the present experimental work was undertaken with the following objectives:

1. To establish and proliferate axenic cultures from juvenile and mature explants.
2. To formulate culture conditions for regeneration and multiplication and regenerant differentiation in somatic tissues.
3. To select the best suited media for direct regeneration.
4. To understand the anatomical processes of shoot bud differentiation under the influence of plant growth regulators.
5. To successfully acclimatize the regenerants using *in vitro* and *ex vitro* hardening methods.

6. To optimize the technique of synthetic seed production and their conversion into plantlets.

7. To study the effects of different days of acclimatization on the physiological and biochemical parameters of regenerated plantlets.

8. To check the clonal fidelity of micropropagated plantlets using ISSR analysis.