CHAPTER 1

INTRODUCTION

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Plants are necessary for the basic existence of mankind as it provide us with food, medicines, fibre, wood and fuel. With increase in population there is an indiscriminate exploitation of plants leading to huge stress on this biotic resource. A number of plant species have become extinct and many are endangered or threatened (Sridhar and Aswath 2011). According to Balick et al. (1996) “even if one population of plant is extinct, all its unique phytochemical germplasm and properties also disappear”. Conventional approaches to germplasm conservation are insufficient to re-establish the endangered plants to their natural habitat and micropropogation strategies and biotechnological methods are imperative for germplasm conservation and sustainable utilization of the plants (Chaudhary et al. 2008).

1.1 Importance of Medicinal plants

Medicinally important plants are a source of primary health care in rural parts of many developing countries (Sen et al. 2014). The high price of synthetic drugs and their related side effects have compelled even the urban population to consume herbal based medicine. India has 2.4% of world land area with 8% global biodiversity and its different forest type account to 90% of medicinal plants. In India it is estimated that there are 25,000 effective plant based formulation and 7800 manufacturing units involved in the production of natural health products and herbal formulations (Aneesh et al. 2009). The importance of these medicinal plants can be assessed as the annual demand for the year 2005-2006 has been estimated as 3, 19,500 metric tonnes which comprises of herbs utilized in domestic herbal industry and rural household (Ved and Goraya 2007). Similarly the Export- Import bank of India reports that the trade of medicinal plants in 1997 values to $ 5.5 billion which is growing rapidly while WHO reports that the international market of herbal products is $6.2 billion which is estimated to grow to $5 trillion by 2050 (Kumar and Janagam 2011). Studies of Aneesh et al. (2009) suggest that India’s share in such vibrant market is only 2% of the trade and good agricultural practises need to be emphasised to increase its share in world market and also prevent depletion of this valuable resource.

The indiscriminate harvesting of the plants for active principle has lead to depletion of many medicinally important plants in natural habitat. Another important factor affecting the status of wild plants used in herbal medicine is ever increasing population of human and livestock (Hu et al. 2005). Conservation of these medicinally important plants is imperative as plants

are effective against several diseases. *Aegle marmelos*, *Argemone mexicana*, *Asparagus racemosus*, *Coleus forskohlii*, and *Rubia cordifolia* are reported to be effective against HIV AIDS (Sabte 2011), *Euphorbia hirta*, *Alternanthera philoxeroides*, *Boesenbergia rotunda*, *Andrographis paniculata*, *Carica papaya*, *Cladogynos orientalis*, *Cymbopogon citrates* etc.on dengue fever (Abd Kadir 2013). Thus these medicinally important plants can be propagated and the active principles can be utilized against several diseases.

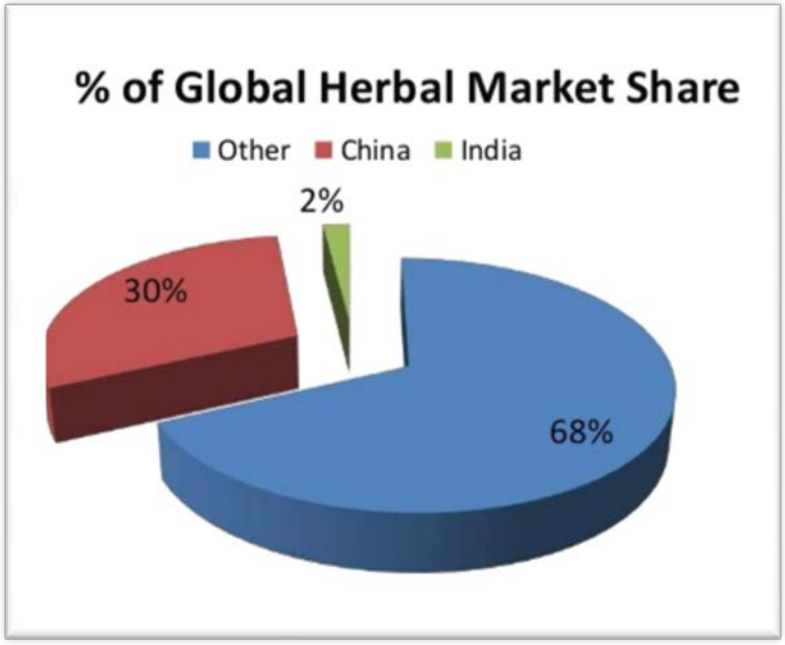

Figure 1: Percentage of global herbal market share (Source: Aneesh et al. 2009)

1.2 Plant tissue culture and its importance

"The history of plant tissue culture has been a long and tenuous one" (White 1943). The remark by Dr P.R White still holds good as the technique of plant tissue culture progressed from the successful attempts for culturing plant cells in medium (Nobe'court 1939; White 1943; Gautheret 1945) to the genetic engineering technology of the 21st century. To carry out any biotechnological intervention it has become imperative to first establish an efficient reproducible protocol for its regeneration followed by the different manipulations. Tissue cultured plants that are genetically engineered provide information on plant molecular biology and gene regulation (Wang et al. 2016). It is utilized for mass production, quality and sterilized condition of the target plant and a tissue culture protocol is a prerequisite before performing molecular work (Ghorpade et al. 2012).The technique encompasses the in vitro regeneration of the plants where clones can be produced from a cell or a plant part (explant) when incubated on a medium of a defined composition (Evans et al. 2003). The regeneration

of the in vitro shoots is achieved by direct or indirect mode of organogenesis. In direct organogenesis the shoot buds are induced directly on the explants while in indirect mode of organogenesis they are differentiated on a callus phase. The success in the generation of disease free identical regenerates depends on a number of factors such as suitable explants, growth regulators which determine the type of response, gelling agents and physical factors such as light, temperature and humidity (Sujatha et al. 2007; Deore and Johnson 2008). Regeneration of a number of plant species from leaf explant have been achieved such as *Abutilon indicum* (Shankar Singh et al. 2016); *Solanum nigrum* (Sridhar and Naidu 2011); *Chlorophytum borivilianum* (Ashraf et al 2014) etc.

Besides the in vitro plants, callus and cell suspension cultures can be utilized for the production of important constituents on a sustainable basis. Calluses are tissues induced by differentiated cells as a response to physical and chemical factors under the influence of determinate hormonal conditions (Mantell et al. 1994). They have the ability to differentiate into tissues, organs or embryo and subsequently regenerate into a whole new plant (Paiva and Paiva 2001; Pierik 1990) Callus can be utilized to study the culture conditions necessary for the explants to grow, cell development, products of primary and secondary metabolism and to obtain cell suspension cultures. The callus and suspension culture can also be used to isolate economically valuable phytochemicals which can prevent the disturbance of natural population (Ogita et al. 2009; Berkov et al. 2009).

Production of calli form from fragments of stems, leaves and roots are mainly carried out to determine the culture conditions required by the explants to survive and grow, study cell development, exploit products coming from primary and secondary metabolism and obtain cell suspension in propagation. It can also pave the way for isolating economically valuable phytochemicals, which can avoid collecting plant materials from natural sources. Callus formation has been reported in a number of plants with different explants and growth regulators. Callus from lamina and petiole in *Anthurium andreanum* (Road et al. 2012), seedling leaf and stem in *Celosia argentea* (Abu Bakar et al. 2014), epicotyl in *Citrus acida* (Chakravarty and Goswami 1999), stem in *Cyclea peltata* (Bhagya and Chandrashekar 2013) roots in Flax (Gomes da Cunha and Ferreira 1996), seed in *Triticum* (Shah et al 2003).

1.3 Role of Plant Growth Regulators (PGR's) in plant tissue culture

Phytohormones or PGR's play an important role in differentiation; organogenesis and cellular morphogenesis. They are a group of naturally occurring organic substances which influences physiological processes at low concentration (Davies 1995). The media is supplemented with

these hormones and accordingly different morphogenic responses are obtained in different species. Gamborg et al. (1976) suggested that PGR's are essential requirement for the success of any invitro development. In 1957, it was demonstrated in tobacco pith tissue that the relative ratio and concentration of cytokinin and auxin could control the organogenetic pathway. High level of cytokinin promoted shoot formation and high auxins formed roots. Equal concentration of both these PGRs caused the tissues to grow in an unorganized fashion (Skoog and Miller 1957).

Two important classes of PGR's are auxin and cytokinin and briefly their role are as follows:

- **Auxins:** Commonly used auxins are 2,4D (2,4-dichlorophenoxy acetic acid) , NAA (1-naphthalene acetic acid), IAA (1H-indole-3-acetic acid) and IBA (1H-indole-3-butyric acid). IAA occurs naturally in the plant tissue. Auxins are responsible for elongation of stem and internode, tropism, apical dominance, abscission and rooting (Razdan 2008). Auxins also promote growth of the callus cell suspension or organ and to regulate morphogenesis (George and Sherington 1984).
- **Cytokinins:** Commonly used cytokinins are BAP (6-Benzylaminopurine), 2-ip (6-γ-γ-dimethyl aminopurine), Kinetin (N-2-furfurylamino-1-H purine-6-amine), Zeatin. Cytokinins are concerned with cell division, modification of apical dominance and shoot differentiation. (Razdan2008). They became an important ingredient of tissue culture medium as it was revealed to have an ability to promote cell division in tobacco tissues (Miller et al. 1955) and developmental role in root and shoot formation (Shani et al. 2006; Dello Ioio 2007).

1.4 Metabolites from plants and their importance

Plants provide natural products which are used in pharmaceuticals; agrochemicals, as flavours, fragrances and organic pesticides (Balandrin and Klocke 1988). These "phytochemicals" are mostly primary and secondary metabolites which are important ingredients of Ayurveda, Unani and Siddha medicines catering to the health care needs of a large population in developing countries such as India. The primary metabolites are carbohydrate, proteins and lipids which are involved in growth and normal functioning of the plants while secondary metabolites are produced as a defence mechanism which are not particularly involved in growth and development (Irchhaiya 2015). The yield of the plant metabolite through conventional method of plant propagation is insufficient to meet the growing demand and Rao and Ravishankar (2002) proposed that biotechnological approaches

especially plant tissue culture provides a viable option for the industrial production of bioactive plant metabolite. Vanisree (2004) has aptly laid down the advantages of using the cell culture for the extraction of these valuable compounds as compared to the conventional cultivation of whole plants as compounds are produced independent of climatic or soil condition, free of microbial infection and automated control of cell growth and metabolite processes reduces the labour cost and increase productivity.

Explants, such as leaves, stems, roots, and meristems can be used for multiplication and extraction of secondary metabolites (Hussain et al. 2012) such as vindoline, catharanthine, vincristine in *Catharanthus roseus* (Ataei- Azimi et al. 2008),β-sitosterol and caffeic acid in *Sericostomapauciflorum*(Jain et al. 2012),iridoids, phenolics, flavonoids and tannins in *Aloe arborescens* (Amoo 2012).

1.5 Mutation and its importance

Mutations are heritable changes which alter the genotype of an individual. The scientific study of mutation started in 1910 when T.H Morgan reported white eyed male mutants in *Drosophila melanogaster* while Auerbach (1946) was the first to induce mutations with chemical mutagens. Consequently mutations are of two types – Spontaneous mutation and Induced mutations. Mutations are induced in seed and vegetative propagated plants by physical or chemical mutagens and the effect is influenced by many factors such as duration of treatment, pH, temperature and treatment method (Kangarasu et al. 2014). Mutagen treatment causes the breakage of nuclear DNA and during the DNA repair mechanism new mutations occur randomly which are heritable (Kangarasu et al. 2014). Spontaneous mutations occur at a very low rate therefore induced mutations provide tools for rapid creation and increased variability within the plants (Maluszynski et al. 1995). Specifically induced mutations have an ability to change one or two traits in a cultivar without altering the remaining genotype (Patade and Suprassana 2008).

Both physical and chemical mutagens are used for inducing variability because of their unique application. Physical mutagens provide accurate dosimetry and uniform penetration of multicellular system while chemical mutagens are preferred because of it high rate of mutation. Physical mutagens generally used for the experimental system are ionising radiations such as X-rays and gamma rays while chemical mutagens are alkylating agents such as Methyl methane sulphonate (MMS), Ethyl methane sulphonate (EMS), Nitrosoguanidine etc., out of which EMS is most commonly used chemical mutagen. The

mode of action of EMS is that it alkylates the guanine bases and causes mispairing such that alkylated G pairs with T instead of C leading to G/C to A/T transition (Bhat et al. 2007).

The major strategy of mutation breeding is to alter one or more agronomic traits which will limit their productivity or increase their quality or potential to induce genetic variability (Novak and Brunner 1992).The first mutant variety was X-ray induced tobacco variety "Chlorina F1" released in Indonesia in 1934 followed by a cotton variety "M.A 9" (1946) developed in India (Maluszynski et al. 2001). Since then more than 2543 mutant cultivars from 175 plant species have been released in 50 countries all over the world (Chopra 2005, Bhat 2007) with China leading with 605 mutant varieties followed by India with 259 varieties (Jain 2005).

1.6 In vitro mutagenesis and its application in plant tissue culture

Mutation breeding programme was successful in introducing many varieties of agronomic traits; however more precision, effectiveness and less time duration are the attributes which are sought after by plant breeders. Maluszynski et al. 1995 suggested that mutation technique along with in vitro culture method can speed up the breeding programme by generation of variability, selection and multiplication of the new genotype.

Conventional mutation breeding poses problems due to large mutagen treated population to be screened and unsatisfactory selection methods. These problems can be overcome by combining the technique of in vitro cultures and mutagenesis which is relatively simple, inexpensive and efficient (Patade and Suprassana 2008).

The advantages of in vitro mutagenesis over conventional mutation are

- high frequency of mutation
- uniform mutagen treatment
- application of selective agents to homozygous population
- single cell can be used compared to a complex whole plant
- less space is required to handle large population of cells
- Obtaining disease free plant material (Evans and Sharp 1983).

The main bottle neck with in vitro mutagenesis is formation of chimaras, which can be controlled by propagating the plants to 3-4 generation (Misra and Dutta 2007).

Mutagenesis had been induced in plants with several mutagen of which EMS is the most common mutagen. It has been specifically useful in floriculture, horticulture and in enhancement of several metabolites. The change in fatty acid profile under the influence of

EMS has been documented in a number of plants. Within *Arabidopsis*, mutants were developed which had fourfold increase in Oleic acid and two fold increase in Palmitic acid (James Jr et al. 1990); and decrease in long chain fatty acids (Kunst et al. 1992). In the Tamla variety of *Brassica napus* the Oleic acid content was again increased to 76.9% (Lee et al. 2014). These reports suggested that fatty acid content in the plants can be altered under the influence of EMS.

1.7 Selection of *Portulaca* spp. as experimental plant

The members of the family Portulacaceae are cosmopolitan generally herbs and shrubs with 15-30 genera and 500 species (Neill 1974). According to Geesink (1969) *Portulaca* is divided into two subgenera: subgenera *Portulacella* (F. Muell.) Legrand with glabrous nodes and flowers in dichasia and subgenera *Portulaca* with nodal scales or hairs and terminal flowers. The presently studied species belong to subgenera *Portulaca*. Both the species are recorded from west of North America, South America and Africa with some representatives in Europe and Asia (Gilbert and Phillips 2000). In the present work the following two spp. of *Portulaca* are selected.

1.7.1 *P oleracea* Linn.

P oleracea is commonly known as Purslane and is a succulent herbaceous erect plant found growing along the roadside, gardens, crop field and other distributed land area. The stem is branched, cylindrical, solid, greenish red and glabrous. The leaves are stipulate, arranged alternately with entire margins, sessile, obovate, glabrous with obtuse apex. Older leaves at the time of shedding develop red margins. The inflorescence is dichasial cymose, flowers are pentamerous yellow and the fruit is a capsule.

P oleracea is antibacterial, -virus, -antherosis, -caducity and enhances immunity. It is useful in headache, stomach ache, painful urination, dysentery, enteritis, mastitis, lack of milk flow in nursing mothers, and in postpartum bleeding, inflammation, skin sores and ulcers. Fresh herb is used as poultice or juice (Grieve 1992).

1.7.1.1*P oleracea*- source of Fatty acids

Chemically fatty acids are carboxylic acid consisting of a hydrocarbon chain and a terminal carboxyl group. The predominant fatty acids are Palmitic acid (16:0), Linoleic acid (18:2) and Linolenic acid (18:3). The number indicate carbon chain length, : indicates unsaturated

bond and the number which follows it is the position of unsaturated bond numbered with the carbon atom of the carboxyl group as C1(Kharenko et al. 2011).

Figure 2: General habit of *Portulaca oleracea* Linn.

Omega fatty acids are essential fatty acids which are not synthesized in human body but need to be ingested either through regular diet or supplementation. They determine the composition of cell membranes and membrane dependent functions such as integral enzyme activity, membrane transport and receptor function; play an important role in prevention and treatment of coronary artery disease, hypertension, arthritis, inflammatory autoimmune disorder and cancer (Simopolous et al 1991). Fishes provide an excellent source of these essential fatty acid but they are associated with potential hazard of heavy metal and fat soluble pollutants like PCBs and dioxin which may accumulate in the food chain **(**Asif 2011). *P oleracea* is a richer source of omega 3 fatty acid within the vegetables examined so far (Simopolous et al 1995; Palaniswamy et al 2001). It contains high level of vitamin A, C, E, and betacarotenes and is extensively included in the Mediterranean diet where the incidence of both cardiac diseases and cancer are low. High content of alpha linoleneic acid in this plant decreases the occurrence of cardiovascular diseases and cancer (Simopolous et al 1991;Liu et al. 2000; Omara-Alwala et al. 1991; Cave 1991; Simopolous et al. 1992). Alpha Linolenic acid is a precursor of long chain omega 3 fatty acids such as eicosapentanoic acid (EPA), docosapentanoic acid (DPA), and docosahexaenoic (DHA) (Brenna et al. 2009). Adequate intake of omega 3 fatty acid is essential for visual functions and neural development (Dyall et al. 2008).

Described as “power food of future” (Simopoulos et al 1995) because of its high nutritive and antioxidant properties unfortunately purslane has not been domesticated or fully evaluated for its nutritive value (Ezekwe et al 1999). *P oleracea* is a viable option which can be utilized as a source of PUFA for vegetarians and its easy availability/affordability can ensure its reach to the poorest of the poor which can be a potential solution to eradicate malnutrition among the population of developing countries.

1.7.2 *P grandiflora* Hook.

P grandiflora commonly known as nine o’clock plant is a succulent herb and was the second species selected for the present study. Morphologically the stem is red, branched and glabrous while the leaves are simple, sessile, alternate to opposite, stipulate lanceolate with acute apex. The flowers are terminal, solitary surrounded by a whorl of leaves. Flowers are arranged in cymose inflorescence, bisexual and perigynous. Different colours of the flowers are reported ranging from white, yellow to red and the fruit is a capsule.

Figure 3: General habit of *P grandiflora* Hook.

P grandiflora is used to cure sore throat, skin rashes and a putative immunostimulant (Chavalittumrong et al. 2007). It is effective on hepatitis B surface antigen (Zheng and Zhang 1990). *P grandiflora* is rich in secondary metabolite, betalains-a pigment which is extensively used in food industry.

1.7.2.1 *P grandiflora*- source of betalains

The food, cosmetics and pharmaceutical industry rely heavily on the colouring material which makes the product appealing to the consumer. With increased awareness about the hazards of chemicals, synthetic dyes are frequently replaced by natural pigments in these industries. Natural pigments obtained from plants are used as colour additives in food, pharmaceuticals and cosmetics (Trejo-Tapia et al. 2008). The stability of natural pigment is a cause of concern and are costlier than the synthetic dyes yet they are being preferred by the consumers indicating that search for new and alternative sources of the pigment is an important area of research (Cai et al. 2005). Common pigments which are used as a colorant are anthocyanins, betalains and caretenoids.

Betalains are food safe colourant, water soluble and need no chemical modification before being used in food and do not provide non desirable flavour to food (Trejo-Tapia et al. 2008). These pigments are present in 13 families of Caryophyllales and some genera of Basidiomycetes (Clement and Mabry 1996). It can be divided into two types-

(i) Red to red violet betacyanin

(ii) Yellow betaxanthin (Moreno et al. 2008; Strack et al. 2003)

Betacyanins are further classified into 4 types based on their chemical structures ie betanin-type, amaranthin- type, gomphrenin- type and bougainvillein- type (Strack et al. 1993).Structurally they consist of betalamic acid which condenses with cyclo DOPA to form red colour betacyanin and with different amino acids and amines to yellow betaxanthin (Moreno 2008; Strack et al. 2003).

In most countries the use of food additive is governed by strict regulation and in United states and European Union only beet root as a source of betalain is exempt from batch certification (Moreno et al. 2008) but it has earth like flavour due to geosmin and high nitrate concentration associated with the formation of nitrosamine may affect its commercial use (Strack et al. 2003; Stintzing and Carle 2007). Beet roots are the main commercial source of betacyanins and the current trend is to search for alternative source of betalains (Cai 2005) of which *P grandiflora* is frequently explored as a suitable choice.

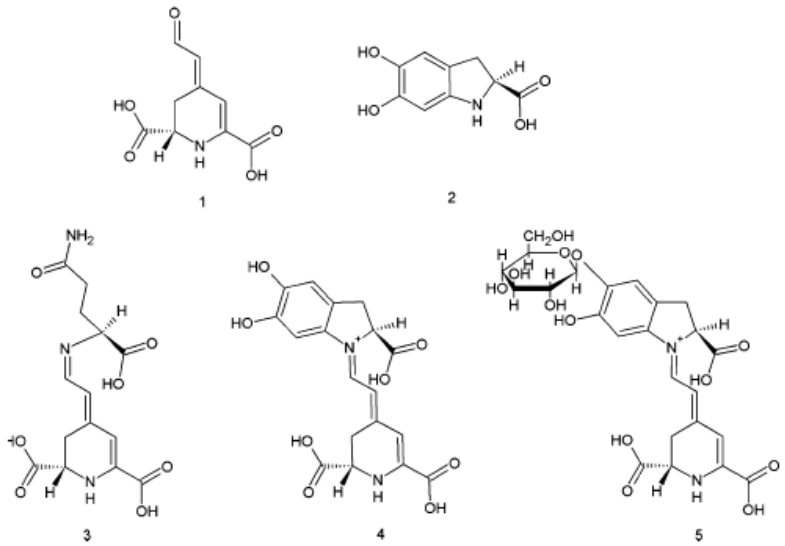

Figure 4: Structural components of betalains (1) betalamic acid (2) Cyclo DOPA (3) vulgaxanthinI (the main yellow pigment of beet) (4) betanidin- basic structural unit of betacyanin (5) betanin -the main red pigment of beet. (Fig courtesy- Georgiev et al 2008)

1.8 Rationale of the selection of *P oleracea* and *P grandiflora* for the present studies

Regeneration in *Portulaca* has been achieved with different explants such as within *P oleracea* it has been reported with nodal, shoot tip and petiole explants (Safdari and Kazemitabar 2009). In *P grandiflora* the explants utilized for the regeneration of in vitro shoots were hypocotyl explants in *Portulaca* sp cv "Jewel" (Bhuiyan and Adachi 2002), Shoot tip (Jain and Bashir 2010), nodal explants (Safdari and Kazemitabar 2009). The leaf explants were used in the study of Safdari and Kazemitabar (2009) for the generation of callus. These studies suggested that in vitro regeneration from leaf explants need to be developed to produce high number of healthy in vitro shoots as the number of shoots reported were less.

P oleracea has been evaluated for its fatty acid content within in vivo plants (Palaniswamy et al. 1997, Ezekwe et al. 1999) and high content of Linolenic acid is reported in the plants. However fatty acid content or its profiling within in vitro cultures has not been reported. Similarly mutation has been induced in *Portulaca* spp. (Gupta 1970; Raghuvanshi and Singh 1979; Wongpiyasatid and Hormchan 2000) mostly with the floriculture point of view but its effect on metabolite profiling has largely been overlooked. In vitro betalain content has been studied in *Talinum triangulare* (Swarna et al. 2013) but *Portulaca* spp. is yet to be explored for its production of betalains from in vitro plants. All these studies indicated that there is a

need to develop a highly efficient reproducible protocol for regeneration and subsequently evaluate its fatty acid content in vitro. The following studies are an attempt to address these issues.

1.9 Hypothesis for the present studies

In vitro regeneration in the *Portulaca* species has been tried but the synthesis of metabolites in shoot and callus cultures need to be exploited. Thus it was envisioned to standardise a protocol for a highly reproducible efficient regeneration system for shoot and callus cultures and analyse them for metabolite content as *P oleracea* is rich in omega fatty acids while *P grandiflora* has a high content of betalains.

Mutations have been known to increase the metabolite content in different species, therefore EMS was selected as a chemical mutagen for regeneration and alteration of metabolite content was evaluated to select high yielding cultures.

1.10 Objectives of the thesis:

The objectives were designed for both the species of *Portulaca* which are as follows:

- Establishment and Optimisation for regeneration of shoot and callus cultures in both the species
- Multiplication and Elongation of shoot cultures for highly efficient regeneration system in both the species
- Fingerprinting of fatty acids within in vivo samples and in vitro cultures of *P oleracea*
- Estimation of betalain content in different samples of *P grandiflora*
- Analysis of EMS treated shoots and callus cultures for fatty acid finger printing in *P oleracea*