INTRODUCTION

Legumes are widely grown throughout the world and their dietary and economic importance is globally appreciated and recognized. Legumes not only add variety to diet but also serve as an economical source of supplementary proteins for a large human population.

Grain legumes are being cultivated in India since time immemorial. They have high total protein content (20-26%) and can be considered as natural supplements to Cereals. Grain legumes provide 220-250 g protein per Kg. Hence, legumes are considered as 'poor man's meat'. Therefore in the present context of our economic development, the exploitation of Legumes in diet with combination of Cereals to make it nutritionally balanced and appears to be the only feasible approach to eliminate 'protein calorie' malnutrition in the near future (Kakati *et al.,* 2010).

The cultivated groundnut (*Arachis hypogaea* L.), an annual herb belonging to the family Fabaceae (Leguminosae), is classified into two subspecies, subsp. *fastigiata* Waldron and subsp. *hypogaea* Krap.et.Rig. The subsp. *fastigiata* contains four botanical varieties, var. *vulgaris*, var. *fastigiata*, var. *peruviana* and var. *aequatoriana.* The subsp. *hypogaea* contains two varieties, var. *hypogaea* and var. *hirsuta.* Each of these botanical types has different plant, pod and seed characteristics (Krapovickas and Gregory, 1994).

Groundnut (*Arachis hypogaea* L.), the king of oilseeds is an important legume grown and consumed predominantly in arid and semi-arid regions of the world. It is a vital food and cash crop resource for poor farmers in Asia and Africa which offers a rich source of oil, protein, minerals (Ca, Mg, P and K) and vitamins (E, K and B1). In India, it occupies an area of 47.66 lakh ha with a production of 47.49 lakh tonnes, which accounts for a productivity of 996 kg/ha (Anonymous, 2014).

The domesticated groundnut *(A. hypogaea)* is currently the fourth most important oilseed worldwide and is considered a key crop in subsistence agriculture in several countries due to the high oil and protein content of it's seeds (Singh and

Singh, 1992). Groundnut seeds are used as a direct source of human food, oil and fodder. In some Asian countries, groundnut oil has also a medicinal value.

Groundnut (*Arachis hypogaea* L.) is an annual warm-season plant of the Leguminosae that originated in South America and it has been cultivated since ancient times. Groundnut seeds contain 44-56% oil and 22-30% protein on a dry seed basis. Large seeded varieties are used for roasting and confections and small seeded types are used for oil.

The major groundnut-producing countries of the world are India, China, Nigeria, Senegal, Sudan, Burma and the USA. Out of the total area of 18.9 million hectares and the total production of 17.8 million tonnes in the world, these countries account for 69% of the area and 70% of the production. India occupies the position both in regard to the area and production in the world. About 7.5 million hectares is put under it annually and the production is about 6 million tonnes. 70% of the area and 75% of the production are concentrated in the four states of Gujarat, Andhra Pradesh, Tamil Nadu and Karnataka.

The groundnut crop has enough morphological, biochemical and physiological variability. It has narrow genetic base because of its monophyletic origin and lack of gene flow due to ploidy and self pollination (Mandal *et al*., 2007). The extent of genetic improvement through conventional breeding methods is very much limited and continued mutation breeding supplement variability and could confer specific improvement without significantly altering its acceptable phenotype (Ojomo *et al*., 1979).

The genetic variability is highly desirable for developing new cultivars, which is induced by mutagen treatments and natural spontaneous changes. The spontaneous mutation rate is pretty low and can’t be exploited for breeding. So, artificial mutations are induced with physical and chemical mutagen treatment. Quite many useful genetic changes have been induced by mutagen treatment including high yield, flower colour,

disease resistance, early maturation etc. have been noticed in several crop and ornamental plants.

Mutations have played a great role in increasing world food security. Since new food crop varieties embedded with various induced mutations have contributed to the significant increase of crop production (Kharkwal and Shu, 2009). Mutations are known to enhance the genetic variability of crop plants and the efforts are being made to improve the genetic makeup of groundnut crop for higher yield, oil content and development of cultivars resistant to diseases and pest.

Mutation breeding has become increasingly popular in recent times as an effective tool for crop improvement and more than 2250 mutant cultivars have been released worldwide (Ahloowalia *et al*., 2004). Artificial mutation induction can be carried out using physical and chemical mutagens and mutation induction with radiation was the most frequently used method to develop direct mutant varieties (Ahloowalia *et al*., 2004). Treatment with mutagens alters genes or breaks chromosomes. Gene mutations occur naturally as errors in DNA replication. Most of these errors are repaired but some may pass to the next cell division to become established in the plant offspring as spontaneous mutations.

The last 30 years have shown mutations to becoming a useful supplementary tool for the genetic improvement of cultivated plants. The FAO/IAEA Mutant Varieties Database contains over 1737 accessions. The mutations are induced artificially by physical and chemical mutagenic agents. Physical mutagens include electromagnetic radiation, such as gamma rays, X rays , UV light and particle radiation such as fast and thermal neutrons, α and ß particles. Radiation and chemical mutagenesis were used widely for producing useful mutants with improved characteristics in peanut and many crops (Rehman *et al.,* 1987).

Physical and/or chemical mutagens cause random changes in the nuclear DNA or cytoplasmic organelles, resulting in gene, chromosomal or genomic mutations. Induced mutagenesis is an established method for plant improvement, whereby plant

genes are altered by treating seeds or other plant parts with chemical or physical mutagens. Voluminous work has been done worldwide for the improvement of both seed and vegetatively propagated crops through induced mutation (Haris *et al.*, 2013).

Gamma rays are used for improving growth and quality of plants, for their high mutation frequency and can interact with atoms and molecules, thus producing free radicals in cells that affect morphology, anatomy, biochemistry and physiology of the plants (Chahal and Gosal, 2002).

Mutation breeding by gamma rays is one of the most powerful methods for developing new varieties, which is very successful in ornamental crops. Radiation mediated morphological, structural and functional changes in a plant are governed by the intensity and duration of the gamma rays which generally induce cytological, biochemical, physiological, morphological and genetical changes in cells and tissues (Rahimi and Bahrani, 2011; Jan *et al.*, 2012; Chandrashekar *et al*., 2013).

The biological effect of gamma radiation is mainly due to the formation of free radicals by the hydrolysis of water, which may result in the modulation of an antioxidative system, accumulation of phenolic compounds and chlorophyll pigments (Kovacs and Keresztes, 2002; Kim *et al*., 2004).

Treatment of crop varieties with gamma radiation has been found to alter the germination and accumulation of proline content resulting in the development of stress tolerant varieties, along with enhancement in the crop yield (Dehpour *et al*., 2011). Chemical mutagens could be successfully applied to induce mutations where no irradiation facility is available. In same case, the efficiency of chemical mutagens has provided to be greater than those of physical mutagens Jacops, (2005).

Ethyl methyl sulfonate (EMS) in an alkylating chemical mutagen and causes high frequency of gene mutation and low frequency of chromosomal aberrations (Van Harten, 1998). At a low concentration, EMS generates G/C to C/G or G/C to T/A transversion, or A/T to G/C transition by pairing errors of 7-ethylguanine and 3-

ethyladenine respectively (Greene *et al*., 2003). Based on codon usage, EMS may also induce nonsense and missense mutations (Callum *et al*., 2000). EMS induced mutagenesis was employed in several species to create genetic variability either *in vivo* and *in vitro* (Kim *et al*., 2003).

Sodium azide (NaN_3) is a chemical mutagen and has been one of the most powerful mutagens in crop plants. The mutagenicity is mediated through the production of an organic metabolite of azide compound. This metabolite enters into the nucleus, interacts to DNA and creates point mutation in the genome. Several factors such as properties of mutagens, duration of treatment, pH, pre and post treatment, temperature and oxygen concentrations etc. influenced the effect of mutagens.

The dose of a mutagen applied is an important consideration in any mutagenesis programme. Generally, it was observed that higher the concentrations of the mutagen greater the biological damage. Azide is perhaps the least dangerous and the most efficient mutagen in that high yields of mutations are achieved at moderate M_1 sterility rates. Although, in some cases it has been reported that treatments with sodium azide, the physiological effects of azide are weak and few chromosomal aberrations are induced and it delays germination and growth. However, azide treated seeds show complete apparently normal growth in M_1 except for M_1 sterility and a high frequency of M_1 chlorophyll chimaeras.

The development of suitable protocol for genetic improvement of the plants using biotechnological methods is the pre-requisite (Rey *et al*., 2000).The development of efficient *in vitro* culture methods facilitated the use of mutation techniques for improvement of both seed and vegetatively for propagated plants.

Plant cell are unique in that they retain totipotency and developmental plasticity in the differentiated state and have the ability to dedifferentiate, proliferate and subsequently regenerate into mature plants under appropriate culture conditions in a hormone dependent manner (Skoog and Miller, 1957). *In vitro* plant regeneration

from cells, tissues and organ cultures is a fundamental process for the application of plant biotechnology to plant propagation, plant breeding and genetic improvement.

In vitro culture techniques constitute an important part of biotechnology and have the potential not only to improve the existing cultivars but also for the generation of novel plants in a comparatively short time compared to conventional breeding. Plant tissue culture has been proposed alternatively as a tool for propagation of useful genotypes (Orton, 1985; Evans, 1989).

The phenomenon of cellular totipotency is great importance in clonal propagation of elite plants (Murashige, 1974) in isolation of desirable mutants at cellular level (Bottino, 1974) and constructing novel desirable plants from somatic cell hybrids (Frearson *et al*., 1973; Sharp *et al*., 1979).

Progress in plant cell, tissue and organ culture has opened up several new possibilities for the induction of genetic variability and the selection of desirable variants (Evans *et al*., 1983). An efficient plant regeneration system is a prerequisite for genetic plant transformation studies using *Agrobacterium tumefacience* (Horsch *et al.*,1985). Progression techniques for plant cell, tissue and organ culture makes its possible to introduce genetic variability and to more easily select for desirable traits (Evans *et al*., 1984).

In addition, plant tissue culture is considered to be the most efficient technology for crop improvement by the production of somaclonal and gametoclonal variants. The micropropagation technology has a vast potential to produce plants of superior quality, isolation of useful variants in well adapted high yielding genotypes with better disease resistance and stress tolerance capacities (Brown and Thorpe, 1995).

Multiplication of desirable genotypes *in vitro* through biotechnological methods has emerged in the late 1980s as a valuable approach to increase plant productivity (Gupta *et al.*, 1993). Many legumes have been clonally propagated from either root or shoot meristems. Establishment of efficient method for plant regeneration is prerequisite for micropropagation of favorable germplasm and for crop improvement

through unconventional breeding methods such as somaclonal variation, somatic hybridization and genetic transformation. The standardization of *in vitro* plant regeneration protocols without intervening callus phase would certainly help in the mass scale propagation of groundnut cultivars.

The lack of an efficient regeneration system in groundnut has slowed the improvement of this species via tissue culture selection and genetic transformation (Eapen and George, 1994). The cultivated groundnut is known to be relatively recalcitrant in tissue culture (Cheng *et al*., 1992; Heatly and Smith, 1996). Although same successful plantlet regeneration has been reported either by organogenesis or somatic embryogenesis, it was necessary to standardize this technique for the cultivated groundnut for experiments on transformation (Eapen and George, 1994). Tissue culture studies in pean ut have been documented including direct and indirect organogenesis (Palanivel and Jayabalan, 2000, 2002; Palanivel *et al*., 2001; Palanivel *et al*., 2002).

Establishment of highly efficient *in vitro* systems for shoot and root induction, plantlet regeneration from callus cultures in important for fundamental studies of organogenesis as well as for applied agricultural studies such as plant breeding.

Somatic embryogenesis is preferred over plant multiplication because is transformation and proliferation potential is very high and the occurrence of chimeric plants can be minimized significantly (Stefaniak, 1994). Plantlet regeneration has been possible through somatic embryogenesis, organogenesis and callus cultures (Changalrayan *et al*., 1994). Plant regeneration *via* somatic embryogenesis occurs in a wide number of plant species including some species of *Arachis* such as *A.hypogaea* (Sellars *et al*., 1990; Baker and Wetzstein, 1992; Durham and Parrott, 1992).

In vitro regeneration of plants *via* somatic embryogenesis promises a higher potential for use in plant propagation and gene transfer (Ammirato, 1984; Parrot *et al*., 1991). Peanut improvement through biotechnology will largely depend on efficient regeneration system. Plant regeneration via somatic embryogenesis has been

reported from mature embryo axes (Mckently, 1991), immature zygotic embryos (Hazra *et al*., 1987; Ozias-Akins *et al*., 1992) and immature leaf (Sabitharani and Reddy, 1996).

Direct somatic embryogenesis from immature zygotic embryos and 50% conversion was reported in groundnut by (Hazra *et al*., 1989 and Ozias-Akins, 1989) indicated problems on conversion of somatic embryos into plantlets. Somatic embryogenesis mostly occurs indirectly via callus phase or directly from early explants. Among the various modes of plant regeneration from *in vitro* cultures, somatic embryogenesis has emerged as most efficient and promising tool for peanut (*Arachis hypogaea* L.) crop improvement programmes (Mckently, 1995).

The combination of plant tissue cultures with mutation induction techniques may be an effective way to crop improvement (Gao *et al*., 1992). However, experience in applying radiations or chemical mutagens to *in vitro* cultured plant material is limited and there are only few reports on successful selection of mutants after *in vitro* application of mutagens (Micke *et al*., 1990). Mutagenesis *in vitro* is an important field for crop improvement (King, 1984). Induced mutations are of great use for plant breeding either directly to improve specific traits or indirectly for cross breeding experiments (Negrutiu, 1990). Plant tissue culture methods are now finding applications of variants and varieties resistant to various mutagens. Induced genetic variability in plant cells by physical and chemical mutagens may help to isolate mutants. Application of physical and chemical mutagens in tissue culture has been reported (Bottino, 1975).

A combination of explant irradiation and *in vitro* regeneration is mostly effective for manifestation of variants (Novak and Micke, 1987). Considerable options led to practicability of the mutagenesis techniques for improving productivity of a vast array of organisms useful to man (Chopra and Sharma, 1985). The application of *in vitro* mutagenesis has vast potential for increasing the available genetic variants in the years to come.

Ethyl methane sulphonate (EMS) is most commonly used chemical mutagen for *in vitro* mutagenesis. A few traits were selected after treatment with this mutagen (Flick *et al*., 1983). N - Methyl - N - nitro - N- nitroguanine (NG), another mutagen has successfully been used in deriving cell lines resistant to aminoacid analogues (Colijn *et al*., 1979).

In vitro mutagenesis I s a combination of *in vitro* culture and mutation induction which provides the opportunity to increase variability of an economically important cultivar is used on plants in developing varieties that are agriculturally and have high productivity potential. Utilization of *in vitro* plant tissue culture techniques increased the benefiting of agriculture and agricultural industries from biotechnological applications.

Biochemical markers become a popular tool in plant genetics and studies utilizing such markers were also initiated (Ramagopal, 1990). Electrophoresis is a technique used to separate and purify macromolecules such as proteins and nucleic acids on the basis of the differences in their size, charge or conformation. SDS Polyacrylamide Gel Electrophoresis (PAGE) is a common type of electrophoretic technique useful for estimating the molecular weight of proteins. The length of the SDS-Protein complex is proportional to the molecular weight of the protein and is separated on the basis of their size. SDS-PAGE are mainly used for the estimation of protein size and purity, determination of protein subunits, monitoring protein integrity and comparison of the polypeptide.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis is most economical, simple and extensively used biochemical technique for analysis of genetic structure of germplasm. Electrophoresis of protein is a powerful tool for identification of genetic diversity.

Amino acids are important for growth regulation and as modulators of growth and cell differentiation, which may be affecting general metabolism and consequently morphogenesis (Basu *et al*., 1989). Amino acids are involved in the synthesis of other

organic compounds, such as protein, amines, alkaloids, vitamins, enzymes and terpenoids (Ibrahim *et al*., 2010).

Proteins are the essential agents of biological function and amino acids are the building blocks of proteins. The diversity of the thousands of proteins found in nature arises from the commonly occurring 20 amino acids. Proteins are polymers of amino acids with each amino acid residue joined to its neighbor by a specific type of covalent bond. Proteins can be broken down (hydrolyzed) to their constituent amino acids the free amino acids derived from them. Of the over 300 naturally occurring amino acids, 20 constitute the monomer units of proteins. All the 20 amino acids are biologically essential.

In recent years, the application of isoenzyme as markers in morphological and regeneration studies has been reported by Franszet *et al*.,1989. Various approaches like karyological analysis (or) isoenzyme markers to assess the genetic stability of *in vitro* derived plants were revealed by Isabel *et al*., 1993. The isoenzymes, peroxidase and esterase patterns of *in vitro* regeneration was reported by Gupta and Srivastava, 1996.

Changes in peroxidase activity precede the appearance of organs noted by Wolter and Gordon, 1975. Marked reductions in the number of biochemical attributes such as starch, protein, amylase, invertase, malate dehydrogenase, peroxidase and phophorylase with a subsequent increase in soluble sugar and amino acids content precede in *in vitro* shoot differentiation process (Verghese and Kour, 1991).

Since long, peroxidases are known to be involved in growth regulation and different biochemical pathways have been proposed to examine their mechanism of action (Goldberg *et al*., 1987). Peroxidase play a key role in the stiffening of the cell wall and processes associated with plant growth through the formation of phenolic cross link (Fry, 1986).

Isoenzymes by allowing the direct study of gene product has been widely used as marker to screen the variability present in the plant population produced through *in vitro* methods. Because the loss or appearance of an isoenzyme band can be indicated the genetic changes induced if any during *in vitro* culture. (Heinz and Mee, 1971).

The biochemical changes have been observed during *in vitro* morphogenesis in different plant species (Stirin and Jacobsen, 1987; Nielson and Hansen, 1992; Stirn *et al*., 1995; Palai *et al*., 2000). Isoesterase (Praminik *et al*., 1995; Krsnik *et al*., 1999), isoperoxidase (Joersbo *et al*., 1989; Samantaray *et al*., 1999; Kanmegene and Omokoio, 2003), catalase (Palai *et al*., 2000; Clung, 1997; Bagnoli *et al*.,1998) and acid phosphatase (Palai *et al*., 2000; Kormutak *et al*., 2003) activities are most commonly used in several species as parameter to monitor the differentiation pathway.

The present programme was undertaken to investigate the mutagenic effects of gamma rays, Ethyl methane sulfonate and sodium azide on various aspects of *in vitro* culture of groundnut. The standardization of *in vitro* mutagenic protocols for groundnut is useful to evolve useful mutants for mutation breeding programmes.

OBJECTIVES:

In view of all the above, the present study was carried out with the following Objectives.

i. To collect the seeds of an important and commonly cultivated groundnut cultivar TMV-7 for *in vitro* culture studies.
ii. To standardize suitable media composition for direct regeneration from seed explants like WEC and WEA.

iii. To select suitable hormonal concentration for callus induction and plantlet regeneration from different seed and seedling explants.

iv. To induce rooting for regenerated shoots obtained *via* direct and indirect plantlet regeneration.

v. To carry out somatic embryogenesis from apical portion of mature embryo with suitable auxins.

vi. To treat the different seed and seedling explants with mutagenic agents like gamma rays, ethyl methane sulphonate and sodium azide.

vii. To know the effect of mutagenic agents in terms of direct, indirect Organogenesis and somatic embryogenesis of groundnut.

viii. To perform biochemical analysis in control and mutagen treated calli derived from immature leaflets and cotyledonary segments in groundnut.

ix. To carry out SDS-PAGE analysis in shoots and calli obtained from control and mutagen treated explants.

x. To analyze amino acids content in shoots derived from mutagenic treatments with whole embryonal axes of groundnut.

xi. To compare the effect of mutagenic treatments on isoenzyme pattern of regenerated shoots.