REVIEW
2. Review of Literature

Plant tissue, cell and protoplast cultures have become useful tools for crop improvement, especially as supplementary means of inducing variability. Tissue culture instability and somaclonal variations are ubiquitous and offer possible benefits as adjuncts to plant improvement (Scowcroft, 1984). The ability to regenerate large number of plants from cultured tissue is important for the successful application of this technology to crop improvement.

The ability to regenerate whole plants is a vital objective of *in vitro* tissue culture, and is necessary for the application of molecular and somatic genetics to crop improvement. Plant regeneration from various experimental materials is the most important component of the tissue culture system, and a critical element in translating the laboratory results into practical application (Morginski and Kartha, 1984).

2.1. Tissue culture media components

A significant factor for the success in tissue culture is the choice of nutritional components and growth regulators. The successful establishment and growth of plant cells *in vitro* generally is determined by the nature of the explant and the composition of the nutrient media. In the last three or four decades a large number of reports have appeared on modification of about two dozen basic constituents.

Nutritional requirements for optimal growth of a tissue *in vitro* may vary with the species. Even tissues from different parts of a plant may have different requirements for satisfactory growth (Murashige and Skoog, 1962). When starting with a new system it is essential to work out a medium that would fulfil the specific requirements of that tissue.

Selection of a culture medium for *in vitro* studies would seem baffling in view of the several well established media and their numerous modifications. Macro and microelement composition of most media is more or less common to all (differing only in their concentrations and Nitrogen source) and any proposed
change pertains mostly to exogenous supply of vitamins, auxins, cytokinins and other growth factors. The choice of a nutrient media is based more on its mineral composition than on its other components. Suitable alterations in the medium composition are warranted depending on the objective of the experiment. A culture medium suitable for callus growth may not be suitable for maintenance of the callus or regeneration or induction of somatic embryos. For example, the high salt media, which are excellent for supporting callus growth and morphogenesis, have not proved very suitable for the growth of excised roots, anthers and other floral organs. Whereas, White’s medium is very good for the culture of excised roots (Street, 1967); Nitsch's (1951) formulation is good for the culture of excised floral organs and the Nitsch and Nitsch (1969) medium for obtaining haploid tissues or embryoids from cultured anthers.

Media constituents

A nutrient medium usually consists of inorganic salts (major and minor) a carbon source, some vitamins and growth regulators. The components of all growth media can be grouped under organic and inorganic constituents.

2.1.1 Inorganic nutrients

The mineral nutrients required for successful growth of plant have been divided by Clarkson and Hanson (1980) into two major groups. (i) Elements like N, P, S that are covalently bonded in carbon components and are vital constituents of the macromolecules, DNA, RNA and protein. (ii) other elements like K, Na, Mg, Ca, Mn, Fe, Cu, Zn, Mo, B etc. that participate in a variety of often overlapping functions including regulation of osmotic and electrical gradients, protein conformation and oxidation-reduction reactions of metalloprotein.

Based on the quantities of requirement media components can be grouped into macro and micro elements

(A) Macroelements: Out of various elements essential for plant growth six elements (N, P, S, Ca, K, Mg) are required in comparatively large quantities and are therefore termed macro or major elements.
(B) Microelements: The microelements usually included in plant tissue culture media are Fe, Mn, B, Zn, Mo, Cu, I and Co.

A brief account of role and mode of supplementation of various inorganic elements in the media is as under:

Inorganic Components

Sulphur: Sulphur is primarily supplied as sulphate (SO$_4^{2-}$) and is utilized for protein synthesis via sulphate respiration as soluble cysteine (99.9%) and a smaller portion as soluble methionine (Giovanelli et al., 1980). Sulphur is also present in such substances as glutathione, believed to be concerned with oxidation reduction reaction in plants. The sulphur requirements of a culture vary depending on the object (0.5 to 10 mM).

Phosphorus: Phosphorus is a structural component of the nucleic acids, DNA and RNA. As a part of the fatty substances, the phospholipids are an essential structural component of the cell membrane. Phosphorus is also involved in all energy-transfer processes in the cell and compounds such as ATP are composed of thee phosphates coupled to a complicated ring structure. Phosphorus is commonly added as PO$_4^{3-}$ at concentrations of 1.1-1.25 mM (Murashige and Skoog, 1962). Due to rapid uptake and interactions with other components (Fe, K, Saccharose), deficiencies may rapidly arise in a medium. In addition, its uptake is influenced by the supply of other elements. For example, Boron deficiency induces a reduction in the phosphorus uptake capacity in Daucus carota cultures (Goldbach, 1985).

Nitrogen: Among all the mineral nutrients, the form of Nitrogen (Oxidized or reduced, organic or inorganic), probably is responsible for the most pronounced effect on growth and differentiation of cultured tissues. Most standard media offer nitrogen as NH$_4^+$ and NO$_3^-$. Individual cultures (Cannabis sativa, Ipomoea, Daucus carota) prefer NH$_4^+$ under certain conditions. Utilization of NO$_3^-$ requires functioning nitrate reductase, the presence of which has by now been demonstrated in numerous callus and suspension cultures (Bray, 1983). Nitrogen supplied in the
form of nitrate ($\text{NO}_3^-$) is readily absorbed by most plants, but the form in which such nitrogen is incorporated into the plant, is highly reduced such as in the amino-group (-$\text{NH}_2$). In a few cases, other sources of N may replace $\text{NO}_3^-$ or $\text{NH}_4^+$ as nitrogen source, or they may augment the existing supply.

**Magnesium, Potassium, Calcium:** The cations Mg, K and Ca play an essential role in cell metabolism. Mg$^{++}$ is one of the essential factors in translation. Its functions include action as co-factor (e.g. glutamine synthase) and activator of various enzymes. Mg is an essential constituent of the chlorophyll molecule.

The Ca$^{++}$ is also essential for deposition of phospholipids and proteins on or within plasma membranes. Its importance is further demonstrated by the efforts of cells to maintain their intracellular concentration at $10^{-6}$ to $10^{-8}$ M even against a concentration gradient using specific Ca$^{++}$ pumps and Ca$^{++}$ binding proteins (calmodulin) located in the cytoplasm and/or individual organelles. Calcium is also a constituent of the middle lamella of the cell wall.

Potassium is supplied at concentration 20mM or higher as the nitrate or chloride, and chloride ions are important in that they stimulate the production of necessary enzymes.

**Microelements:**

The microelements Fe, Mn, Zn, Cu, Mo, I, B and Co act as co-factors and as inducer of enzyme synthesis. Boron is essential for membrane function, permeability and integrity thereby influencing membrane fixed processes, membrane potential and phytohormone metabolism. Lack of iron results in increased contents of DNA and free amino acids, as well as a reduced RNA content. In order to maintain a minimum supply of Fe it is therefore usually added in complexes with EDTA or sequestrin. This also facilitates uptake over a broad pH range, which varies depending on the content of phosphate, NO$_3^-$ and NH$_4^+$ in the medium.

These eight elements take part in catalytic processes going on in the cell. Copper is a part of certain oxidative oxidase which serve to oxidise phenolic
substances. Iron functions as a respiratory electron carrier through compounds such as cytochromes and the oxidative enzymes, peroxidase and catalase. The exact role of Boron in cell metabolism is rather obscure, though implicated in sugar transport in speeding up the rate of sugar movement in the plant.

II. Organic nutrients

Three groups of organic nutrients are required in tissue cultures,

1. Carbohydrates
2. Vitamins
3. Plant growth regulators

1. Carbohydrates - A variety of carbon sources is used in tissue culture media. Cell cultures are usually cultivated heterotrophically and, in most cases, carbon must be added in the form of carbohydrate. The disaccharide, sucrose being the most common. Sucrose is a necessary component in the culture media as most tissue cultures are not autotrophic. Sucrose as a precursor in reduced concentrations, serves to minimize the quantum of alcohol production. It also acts as the main osmoticum, besides being an energy source.

While sucrose is incorporated in the medium usually in the optimal range of 2-4% (W/V) for growth and morphogenesis of most tissues, there are reports that organogenesis in individual cases may require altered levels of sucrose (Narayanswamy, 1994). Some tissues may prefer specific sugar such as maltose, glucose and fructose or sorbitol.

2. Vitamins - The medium requires to be supplemented by one or more vitamins of the water soluble B-complex for healthy growth of tissues in culture. They are apparently synthesized in sub-optimal quantities by callus tissues. Vitamins play a catalytic role in cell metabolism apart from being a factor in accessory food supply, but their requirements vary from species to species.

3. Plant growth regulators : In addition to the nutrients, it is generally necessary to add hormones. The supplementation of phytohormones are necessary to trigger cell division in a tissue explant and sustained growth of the cultures. However,
the requirement of these hormone varies considerably with the tissue, and it is believed that it depends on their endogenous levels.

The growth process of a culture is decisively affected by the ratio of auxins to cytokinin. It is widely believed that plants regulate the levels of active auxins and cytokinins by both synthesis and conjugation. In general, higher auxin and low cytokinin concentrations stimulate cell division, while low auxin and high cytokinin concentrations stimulate cell growth. However, an excessive supply of gibberellic acid and phenolic compounds cancels this balance.

There are five known classes of growth substances Auxins, Cytokinins, Gibberlins, Ethylene, Abscisic Acid (Narayanswamy, 1994). However, only two (Auxins and Cytokinins) are widely used in the culture media.

**Auxins** - Growth substances based on the indole nucleus are referred to as auxins. Auxins may initiate or promote cell division from tissues cultured *in vitro*, can stimulate shoot growth, control vascular system differentiation, regulate apical dominance, delay senescence, promote flowering. The growth and viability of cultures are usually ensured by adding synthetic (2,4-D, NAA) or naturally occurring (IAA) auxins.

**Cytokinins** - Cytokinins are 6- substituted purine compounds. They are now fully recognised as one of the major groups of endogenous plant hormones. In tissue culture media, cytokinins are incorporated mainly for cell division and differentiation of adventitious shoots from callus (Bhojwani and Rajdan, 1983).

Cytokinin is arbitrarily defined in terms of its capacity to promote *in-vitro* cell division and growth of callus tissues in the same manner as kinetin stimulates growth of cultured cells. In addition to the natural cytokinin, such as, Zeatin (r-hydroxy methyl-adenine) and 2-ip (isopentyl-r,r-di-methyl-allyl), synthetic products such as kinetin (6-furfurylaniinopurine), 6-BAP (N6-benzylamino purine) are also used.

**Culture media pH**: The pH of the medium greatly influences the uptake of ingredients, solubility of salts and gelling efficiency of agar. Although the pH of
the medium is altered during culture, an initial pH is selected before autoclaving. The pH is adjusted using NaOH or HCl. A pH of 5.6-5.8 has been found suitable for maintaining all the salts in a near buffered form.

Solidification of Medium: Because of improved oxygen supply and the developing chemical gradients, cultures on solid media are often preferred to liquid cultures. For this purpose, substances with a strong gelling capacity are added to the liquid medium. These reversibly bind water and thus ensure the humidity of the medium desired for culturing, depending on the concentration. An inert natural plant product agar is used very commonly in tissue culture experiments as solidifying agent.

2.2. In vitro plant regeneration

2.2.1. Callus induction

Aseptic culture of the damaged region on a defined medium can cause cell division which can be stipulated and induced to continue indefinitely through the exogenous influence of the chemical constitution of the culture medium. The result is continually dividing mass of poorly differentiated and disorganised plant cell aggregates termed a callus.

In morphological terms it can vary extensively, ranging from being very hard/compact where the cells have extensive and strong cell to cell contact, to being friable where the callus consists of small, disintegrating aggregates of poorly associated cells and has a rather crumbly or creamy appearance. Friable callus is generally most sought after as it is usually the fastest growing and most uniform type and is best suitable for the initiation of cell suspension cultures. Callus morphology is often dependant on explant but can be altered by the modification / supplementation of the growth substance to the culture medium.

Due to its size and nature, callus cultures have an inherent degree of heterogeneity. As there is a unidirectional supply of nutrients (from the medium below) and gases and light (predominantly from above), chemical and physical
gradients will be present within the callus mass. While, in some instances, this heterogeneity is a disadvantage (e.g. in the production of uniform biomass) it may also be an important factor influencing the developmental response of the callus in plant regeneration (Hall, 1991).

Influence of cell origins and genotype on callus type: In normal practice callus cultures are established from multicellular pieces of vegetative tissues. These fragments may be relatively homogeneous with respect to a particular cell type, for example the vascular cambium, storage parenchyma, cotyledons or mesophyll. In contrast, the explant such as stem, hypocotyl, whole embryo may be extremely heterogeneous and contain a wide range of cell types. A tissue such as a piece of stem or hypocotyl is typically a complex of differentiated cell types with differing sensitivities to imposed stimuli. The act of bringing such a heterogeneous fragment into contact with a complex medium, designed to promote callus formation, may simulate the wide variety of cells with differing proliferative capacity, ploidy level, and physiology within the population.

2.2.2 Sub culture

Wounding induces proliferation at the damaged surface of an intact plant which may result in the formation of a callus. Such a callus usually persists for only a short time and rapidly becomes infiltrated with polyphenolic substances, which seal off the wound from the environment. In order to sustain cell proliferation it is necessary to remove the developing callus and place it in culture in the presence of growth promoting substances.

Changes during proliferation and growth: Plant tissue cultures tend to lose the ability to differentiate with successive sub-cultures. Some cultures apparently lose the ability to differentiate and produce recognizable structures, but this can be restored by a change in the hormonal balance in the medium. Skoog and Miller (1957) has shown that an apparently unorganized tobacco callus can be induced to form either more callus or roots or shoots simply by effecting minute changes in the auxin : cytokinin concentration to which the tissue is exposed in the culture medium.
Chaturvedi and Mitra (1975) have demonstrated a shift in the morphogenetic pattern in callus tissue during prolonged culture. Stem callus only produced shoots in short term culture but began to produce embryoids after prolonged culture. It have been shown that embryogenic potential of carrot callus declines with time and this may be altered by changing the composition of the medium. The ability to differentiate into roots and shoots may also be lost with time in culture. Wilson and Street (1975) have shown that freshly initiated culture of *Hevea brasiliensis* will produce roots spontaneously but this property is lost during serial sub-culture.

**Biochemical behavior during proliferation**: Metabolic patterns in calluses may be modified during culture. The isoenzyme patterns of several proteins in *Phaseolus vulgaris* changed during a growth cycle. For examples glutamate dehydrogenase change from a pattern of five to a single electrophoretic band after sub-culture and then gradually returned to five towards the end of the culture period. (Arnison and Ball, 1974).

The failure of tissue culture to accumulate particular compounds implies not a loss of bio-synthetic potential but merely a failure to realize such potential under the conditions used. Key enzymes in a biosynthetic sequence that are observed in the initial isolate may be lost during serial sub-culture or at least are present in such small amounts as to reduce the flow of materials through the pathway below a detachable level. The effect of such lesions may be to lead to an accumulation of intermediates in unusual quantities, or to divert precursors to the synthesis of products not normally encountered, and these appear to be in the minority. In other cultures, the components of a particular biosynthetic pathway persist and the synthetic potential is preserved through many sub-cultures.

2.2.3. Field Transfer

*In vitro* regenerated plantlets often show marked physiological and morphological differences as they are grown under conditions which differ widely from those of seedlings. These include high relative humidity, low light intensity, heterotrophy due to high sucrose concentration, poor aeration and hormonal imbalances. This situation leads sometimes to poor vascular connection through
out the plantlet which is essential for its survival in the field. Xylem vessels sometimes close before rooting and the lack of vascular connections cause poor survival on transfer to the field (Grout and Aston, 1977).

Rooting of *in vitro* shoot often affects plant survival. Two patterns of root formation has been recognized. One consists of direct development of root primordia from cells associated with or in close proximity to the vascular system. The other is an indirect process where root is formed from callus tissue without any vascular connection to stem (Sahay and Verma, 2000).

The proper development of leaves is one of the most important factors for survival in the field. Wetzstein and Sommer, (1982) observed that *in vitro* plants showed reduced palisade parenchyma and increased mesophyll air space and inadequate stomatal closure in water stress. A few days after transfer to the field the leaves resembled those of seed raised plants. This may be one of the reasons for the requirements of acclimatization procedures to adopt tissue culture plants to low humidity in the field.

2.3 Plant regeneration

Plant regeneration *in vitro* occurs via two development pathways: Embryogenesis and organogenesis which differs in initial formation of either a bipolar or unipolar structure.

In somatic embryogenesis a new individual with a bipolar structure (*i.e.* a rudimentary plant with a root/shoot axis) arises from a single cell and shows no vascular connections with its maternal tissue (Haccius, 1978). In contrast, shoot organogenesis is characterized by the production of a unipolar bud primordium with its subsequent development into a leafy vegetative shoot. The developing shoot establishes conducting connection with the maternal tissue. The shoot then becomes rooted *via* root primordia formation and subsequent root organogenesis (Brown and Thorpe, 1986).

2.3.1 Organogenesis

Plant regeneration response has been shown to be under genetic control is several crops such as red clover (Keyes *et al.*, 1980), alfalfa (Hernandez-Fernandez
and Christie, 1989), Sunflower (Sarrafj et al., 1996). The evidence indicates that morphogenetic competence \textit{in vitro} is dependent on the culture environment and the physiological and organizational state of the cells (i.e. callus, cells and protoplast) (Cornejo-Martin et al., 1979, Vasil, 1985). Different factors control morphogenesis in vitro and no theory clearly explains all the responses till date (Sahay and Verma, 2000).

Several studies have shown that success in \textit{in vitro} organogenesis is largely dependent on following factors:

(a) Explant  
(b) Genotype  
(c) Medium composition

Manipulation of these factors leads to the initiation of organized development and ultimately, to dramatic structural changes.

(a) Explants: Plant consists of a heterogeneity of cell phenotypes which arise as a consequence of differentiation and development. This heterogeneity is evident from observation on tissue history, DNA (qualitative and quantitative) and studies on gene expression as manifested by changes in protein and isozyme production during differentiation and development (Nagl, 1978, 1979; Raghavan, 1983).

Changes in gene expression, and hence cell phenotype, may have major influence on a cell's capacity to produce a callus or regenerate plants \textit{in vitro}.

Wide variety of plant parts have been used as the tissue source for cultures by various authors. The type of explant used to initiate \textit{in vitro} cultures appears to be a critical factor in determining the capacity of cells and tissues for regeneration (Maddock, 1985; Vasil, 1987).

Several factors have been identified that are important for explant selection (Murashige, 1974; George and Sharrington, 1984). Some of the factors which affect callusing are as follows:

- the organ serving as explant and its physiological and ontogenetic age  
- the season of explant excision
• the size of the explant
• Explant orientation on media
• pretreatment
• inoculation density etc.

In forages, fast growing meristematic tissues derived from embryos and seedlings are reported to be more responsive in cultures than those from the mature plants (Novak and Konecna, 1982; Lu et al., 1982a,b; Ahuja et al., 1983). In graminaceous species consistent shoot formation has been obtained mainly from callus induced from very immature material such as zygotic embryos and young inflorescences (Maddock, 1985; Vasil and Vasil, 1984; Vasil, 1985, 1987). Young inflorescences were more responsive than seeds in Poa pratensis for regeneration (Van der Valk et al., 1989).

In Indigofera teysamni it was found that high dose of BAP (upto 5 mg/L) induced more callus from leaf and shoot explants of seedlings. However, no such response was seen in mature leaf disc explant. The frequency of shoot production depends upon the physiological age of the explant, the older the tissue, the lower the frequency of shoot production (Ayyappan and Rajkumar, 1988). The importance of the age of explants in determining the morphogenetic expression of pea leaflet has also been emphasized (Morginski and Kartha, 1981).

(b) Genotype: The genotype is one of the major factors in determining the organogenetic response. Species, cultivar and plant dependent regeneration has been reported in many species. A large number of legumes show genotype-specific regeneration as indicated in studies on Medicago (Bingham et al., 1975; Phillips, 1983; Mitten et al., 1984); Trifolium pratense (Keyes et al., 1980; Bhojwani et al., 1984; McLean and Nowak, 1989); Trifolium sp. (Webb et al., 1984); Pisum (Malmberg, 1979, Kunakh et al., 1984); Cajanus (Kumar et al., 1983, 1984 a, b); Vitis (Clog et al., 1990); Allium (Rauber and Grunewaldt, 1988). Individual genotype of the same alfalfa variety have been shown to require different phytohormone and salt concentrations for regeneration (Kao and Michayluk, 1981).
Culture medium composition: A successful organogenesis in vitro involves medium optimization as one of the first steps as there is no single medium that must be used for a given species or type of culture. The chemical composition and physical make up of the nutrient medium are determining factors in plant regeneration.

The ratio of auxin and cytokinin has been reported by various authors to play a significant role in organogenesis. Classical work of Skoog and Miller (1957) suggested that quantitative interactions between growth regulators, especially auxin and cytokinin and other metabolites provide a common mechanism for the regulation of all types of growth including organ formation. Manipulation with stem pith-derived tobacco callus showed that a high ratio of auxin to cytokinins in the nutrient medium favoured root formation, the reverse favored shoot formation, and an intermediate ratio promoted callus proliferation. However, this approach can not be demonstrated in all species. To induce plant regeneration, calli are generally transferred to media with lower auxin levels, which may be further improved by the addition of cytokinins. Further, studies confirm the role of endogenous auxin-cytokinin ratio in organ control. Transposon insertions which inactivate T-DNA genes 1 and 2 involved in auxin biosynthesis decrease the auxin/cytokinin ratio in the tissue and shoots and root forming teratomas are formed as a consequence (Garfinkel et al., 1981; Inze et al., 1984). No callus was induced in Vitis when BA was absent which indicate that BA is required for shoot initiation (Clog et al., 1990). Inactivation of a single T-DNA gene involved in cytokinin biosynthesis increases the auxin / cytokinin ratio and root farming teratomas are produced (Garfinkel et al., 1981; Akiyoshi et al., 1984). In Cajanus cajan the relative effectiveness of different cytokinins for multiple shoot formation was found to be in order - BAP - Kinetin - Zeatin - Adenin (Shiva Prakash et al., 1994), while BAP was found to be better than 2-ip for callus induction in Indigofera (Ayyappan and Kumar, 1989). IAA and IBA were ineffective at all concentrations in inducing roots, whereas NAA showed better response in Bixa sp. (Sharon and D’Souza, 2000)

No influence of the amount of NAA and IAA on explant response was seen in Allium species (Rauber and Grunewaldt, 1988). In two legumes, Arachis and
frequency of shoot regeneration was dependent on the type of auxin present in the medium (Eapen and George, 1993).

2.3.2. Somatic embryogenesis

Somatic embryogenesis can be defined as the process in which a bipolar structure arises through a series of stages characteristic for zygotic embryo development and having no vascular connection with the parental tissue (Ammirato, 1987; Terzi and Loechiavo, 1990; Raemakers et al., 1995). The development of somatic embryos closely resembles that of zygotic embryos both morphologically and temporally. However, Somatic embryos, in contrast to zygotic embryos, grow and differentiate continuously, apparently activating the shoot and root apical meristem with no obvious quiescent state (Zimmerman, 1993).

The similarity between zygotic and somatic embryogenesis is both striking and remarkable. The fact that structurally and developmentally normal embryos can develop from somatic cells indicate that the genetic programme for embryogenesis are totally contained within the cell and can function completely in the absence of gene products from the maternal environment (Zimmerman, 1993).

Somatic embryos have a bipolar structure in which shoot and root meristems are directly connected with no interruption by non differentiated callus tissue (Lorz et al., 1988). Although plants regenerated through tissue culture in some species are less variable than their original donor or explant sources (Feher et al., 1989; Gmitter et al., 1991), enhanced variability (both phenotypic and cytological) from embryogenically regenerated plants over organogenetically regenerated plants from the same explant source have been documented (Armstrong and Phillips, 1988; Browers and Orton, 1982; Ahloowalia and Maretzki, 1983; Karp and Maddock, 1984). The variability may be caused by a constant mutation rate per cell generation with a multiplicative effect due to an increased number of generation in vitro (Peschke and Phillips, 1992).

In somatic embryogenesis, the embryoid is often derived from a single cell although evidence for a multicellular origin has also been obtained. Single cell
origin has been observed for pearl millet (Vasil and Vasil, 1982). Sugarcane (Ho and Vasil, 1983). Multicellular origin has been reported in maize (Vasil et al., 1985), Celery (Browers and Orton, 1982). Somatic embryos have been an excellent source for secondary embryos. It is associated with less of integrated group control of cells organised in the somatic embryos. Some cells break away from group control and initiate new somatic embryos (Williams and Maheswaran, 1986).

The first observations of in vitro somatic embryogenesis were made in Daucus carota (Reinert, 1958; Steward et al., 1958). Since then somatic embryogenesis has been described in more than 200 species (Evans et al., 1981; Tulecke, 1987; Raemakers et al., 1995). Virtually every plant organ has been shown to form embryos.

Embryos of the first recognizable stage, globular stage, generally grow out of cell cluster within 5 to 7 days after transfer to auxin free medium in carrot. After 2 to 3 more days of isodiametric growth, the globular stage is followed by an oblong stage (Schiavone and Cooke, 1987), which signals the shift from isodiametric to bilaterally symmetrical growth and the beginning of the heart stage. This transition is clearly marked by the out growth of two cotyledons, the elongation of the hypocotyl and the beginning of the radicle. By three weeks after induction, plantlets can be identified (Zimmerman, 1993).

In most cases somatic embryos develop up to pre-embryonic masses (PEM's) or globular embryos, without differentiation into organs, before they are subjected to secondary embryogenesis (indirect embryogenesis). In other cases embryos develop up to maturity (direct embryogenesis) (Raemakers et al., 1995).

The nutritional requirements for somatic embryogenesis are not well understood. They are neither specific nor exclusive, since various recipes produce similar results. However, auxin and reduced nitrogen are believed to be the prime controlling factors in somatic embryogenesis. (Laxmi et al., 1999).

Evans et al., (1981) and Sharp et al. (1980, 1982) proposed the concept that somatic embryogenesis is initiated by either of two cell types "pre embryogenic determined cells" (PEDCs) and "Induced embryogenic determined cells" (IEDCs).
PEDCs are already determined for the embryogenic pathway and await only the synthesis of an inducer (or removal of an inhibitor) to resume independent mitotic divisions and express their embryogenic potential. PEDCs are found in embryonic tissues, in the nucellus and embryo within the ovules. IEDCs require redetermination to the embryogenic state, generally by exposure to specific growth regulators. IEDCs occurs in anthers cultures and in callus cultures particularly after treatment with auxins. Once the embryogenic state has been induced there appears to be no fundamental difference between IEDCs and PEDCs.

The potential application of somatic embryogenesis in plant improvement depend to a large extent on whether proliferation is from PEDCs or IEDCs. Direct somatic embryogenesis from PEDCs without cellular destabilization and redifferentiation appear to produce relatively uniform clonal material. Indirect pathway through IEDCs where an intervening destabilized callus phase is present tends to generate a higher frequency of somaclonal variants (Williams, 1987).

**Hormonal role in somatic embryo development**: Many authors (Schiavone and Cooke, 1987; Michalczyk *et al.* 1992; Zimmerman, 1993) have shown that auxin appears to play important roles both in the induction of embryo development in culture and in the subsequent elaboration of proper morphogenesis in embryo development.

*In vitro* development of somatic embryos in carrot was reported to be a two step process, each requiring a different medium. The callus is initiated and multiplied in an auxin rich medium called ‘Proliferation medium’, in which callus differentiates localized groups of meristematic cells called “embryogenic clumps” (EC). In repeated subcultures on the proliferation medium the ECs continue to multiply. However, if the ECs are transferred to a medium with very low auxin or no auxin, they develop into mature embryos. Thus, proliferation medium could be regarded as the induction medium for somatic embryogenesis (Snug and Okimoto, 1981) and each EC a disorganised embryo (Bhojwani and Razdan, 1983).

The promotive effects of NAA (*Lazzeri et al.*, 1987); 2,4-D (*Hazra et al.*, 1989) and picloram (*Kysely and Jacobson*, 1990) on *in vitro* induction of somatic
embryos in cultures of legumes have been demonstrated (George and Eapen, 1994). In carrot, Halperin (1970) reported initiation of embryogenesis by BAP and Gibberellins. However, Fujimura and Komamine (1975) recorded promotive effect of cytokinins on embryogenesis.

The role of exogenous auxin in somatic embryo induction depends on the nature of explant (Ammirato, 1983). The exposure duration of exogenous auxin vary with explant source and species (Dudits et al., 1993). Raemakers et al., (1995) while reviewing primary and secondary embryogenesis opined that in general, auxin and or auxin/cytokinin supplemented media are used in somatic embryogenesis of Gymnosperms and monocot Angiosperm species. In certain, dicot species also growth regulator free and/or cytokinin supplemented media can initiate embryogenesis.

**Gene expression during Somatic embryogenesis**: The dramatic transition from unorganized callus cell growth to somatic embryo development suggest that a substantial reprogramming of gene expression, presumably occurring at the transcriptional level, dictates the development switch (Fujimura and Komamine, 1980; Zimmerman, 1993).

Several genes that are preferentially expressed in somatic embryos belong to a class of hydrophillic proteins called Late Embryogenesis Abundant (LEA) proteins (Dure et al., 1989; Galiba et al., 1986). Most of the LEA transcripts increase significantly in somatic embryos at the heart stage (Kiyosue et al., 1992, 1993; Wurtele et al., 1993).

It was reported that some extracellular proteins (EP) are secreted that could further induce somatic embryogenesis (De Jong et al., 1992; Smith and Sung, 1985). It has been suggested that these secreted proteins play a role in the regulation of cell expansion / which is critical to the maintenance of the integrity of epidermal layer in embryos and to the proper establishment of shape and form (Van Engelen and De vries, 1992; Sterk and De vries, 1993).
2.4 Somaclonal variation:

Assembly of genetic variability is vital for improvement of crop plants. Somaclonal variation can be utilized for the improvement of specific traits, particularly where they are lacking in available germplasm. Plant cell culture has provided a new and exciting option for obtaining increased genetic variability relatively rapidly.

Somaclonal variation was defined by Larkin and Scowcroft (1981) as the genetic variation displayed in tissue culture regenerated plants and their progeny. Peschke and Phillips (1992) while reviewing the subject included in it any genetic, cytogenetic or molecular changes produced during tissue culture or plant regeneration. The somaclonal variation, along with the corresponding changes observed in tissue culture per se has been documented by numerous authors in a large number of species (Karp, 1995; Peschke and Phillips, 1992; Evans and Sharp, 1986). Tissue culture regenerated variants have also been called calliclones (Skirvin and Janick, 1976), pheno variants (Sibi, 1976), protoclonal clones (Shephard et al., 1980) and subclones (Cassells et al., 1991). The utilization of new genetic variability induced either spontaneously or artificially during the culture process has become one of the major objectives of tissue culture.

The widespread occurrence of somaclonal variation in wide variety of plant species has been extensively documented. Somaclonal variation does not appear to be species or organ specific and many of the plant traits for which genetic variability is generated during tissue culture cycle are of agronomic value and can thus provide a valuable adjunct to plant improvement.

Scowcroft (1984) emphasized that plant tissue culture per se appears to be an unexpectedly rich and novel source of genetic variation. The first substantive example of somaclonal variation was recorded in sugarcane in Hawaii (Heinz and Mee, 1969). Variation was observed in morphological, cytogenetic and isozyme traits (Heinz, 1973). Subsequently somaclones were identified with increased resistance to various diseases such as Fiji disease virus, downy mildew and eye spot disease (Krishnamurthi, 1974; Krishnamurthi and Tlaskal, 1974; Heinz et al., 1977), for high sucrose concentration (Heinz and Mee, 1971; Heinz et al., 1977). In
potato, variants have been reported for growth habit, tuber colour, maturity date, tuber uniformity and disease resistance (Shephard et al., 1980). In some cases the somaclonal variants have been field tested and found unstable (Larkin and Scowcroft, 1983), while in other cases stable variants have been selected (Krishnamurthi, 1982) including lines resistant to Fiji disease and downy mildew.

In large number of cases the variants were not useful mainly because of following reasons - negative variation, not novel variation, unstable variants which change after selfing or crossing. This is a novel means of making beneficial selections. However, while serving as a resource seeking desirable traits, it often serves as a nuisance to those attempting to create defined changes in plants through transformation or who wish to preserve germplasm in vitro. The tool has been most successful in crops with narrow genetic bases, where it can provide a rapid source of variability for improvement (Karp, 1995).

The study of the heritability of somaclonal variation has revealed that it can result from either genetic, epigenetic on nongenetic change (Meins, 1983). Non genetic variation includes chimeral breakdown, physiological effects, and the elimination of virus or virus like agents (Cassells, 1985).

Genetic variants are heritable variants as judged from studies on Mendelian inheritance of variant traits (Orton, 1984; D’Amato 1985). Such variants can be exploited for further studies.

Epigenetic change results from a change in gene expression and not gene mutation (Chaleff, 1981; Meins, 1983). Such variants, therefore, revert when sexually propagated. Epigenetic variation can be transient or temporary in later generations even when the material is asexually propagated. This variation includes phenotypic changes that involve expression of specific genes (Hartman and Kasler, 1983). Because explants adopt to an in vitro environment in stepwise fashion by becoming more juvenile, the resulting calli may vary in maturity from juvenile, to fully mature. Plants regenerated from these tissues also vary depending on the developmental stage progression of the tissue when the stimulus to regenerate is applied (Skirvin et al., 1994). Shoot regeneration from dedifferentiated callus can produce an immature, unstable clone that may
eventually revert to the original parental clone. Examples of epigenetic variation include partial fertility, male sterility, or transient dwarfism (Mc Pheeters and Skirvin, 1989; Moore et al. 1991) that are associated with a carryover of growth regulators from the tissue culture medium, tissue or cellular habitation involving the loss of auxin, cytokinin or vitamin requirements by callus (Skirvin, 1978; Meins, 1989; Jackson and Lyndon, 1990).

Somaclonal variation results from both preexisting genetic variation within the explants and variation induced during the tissue culture phase (Evans et al., 1984). Preexisting variation can evolve from non-uniformity in multicellular explants (multiple types of cells such as phloem, parenchyma, cortex and xylem parenchyma).

Factors affecting occurrence and frequency of somaclonal variation:

It can not be said that in vitro culture will always give rise to variation. In fact, a number of factors are identified that influence occurrence and frequency of variations. These include: Explant source, Explant genotype, Culture age, Culture conditions, Ploidy level and Karyological aberrations

(1) Explant source: The source of explant has been considered most often as a critical variable for somaclonal variation (Skirvin et al., 1994). Since all explants are not equal in term of regenerability, it is likely that different selective pressures would be exerted against different explants. This could result in different frequencies and spectrums of somaclonal variation among plants from different explants. Thus, differences in both the frequency and nature of somaclonal variation have been reported when regeneration is achieved from different tissue sources. However, while reviewing the topic Peschke and Phillips (1992), preferred to make two generalizations.

(1). Meristems cultured without a state of differentiation will produce little or no variation compared to when a dedifferentiated state is induced (Karp and Bright, 1985; Bayliss, 1980; D'Amato 1985; Potter and Jones, 1991). Highly differentiated tissues (roots, leaves and stems) produce more variation than explants with preexisting meristems (axillary buds and shoot tips) (Duncan, 1997).
(2) Differences in the stability of tissue cultures produced from different explants can often be traced to variability preexisting in the explant. The most recognized case is polysomaty (wherein diploid and polyploid cells coexist in the same tissue), that may be found in over 90% of plant species (D’Amato, 1985, 1989). Van der Bulk et al. (1990) reported a high degree of polyploidy (58%) in plants regenerated from cotyledon, leaf in Tomato. They also demonstrated that the hypocotyl is polysomatic whereas the other explants tested (cotyledon and leaf) showed diploid cells. Similarly, Sree Ramulu et al. (1986) found that higher protoplast-derived potato plants showed a higher frequency of chromosomal and phenotypic alteration when the protoplasts were obtained from a chromosomally variable cell suspension than when they were freshly isolated from diploid tissue. In tobacco, 0.1-1.87% of the variation was present originally in the mesophyll protoplast, whereas 1.4-6.0% of the variation was attributed to tissue culture stress.

(2) Genotype of Explant: The genotype of plants used for somaclonal variation is an important variable which can influence both the frequency of regeneration and somaclones (Evans et al., 1981; Evans and Sharp, 1986). Frequency of polyploid regenerants in 18 varieties of rice were compared and multiploids were recovered from the indica varieties but not in the Japonica varieties. Similarly, the frequency of chlorophyll deficiency mutants varied significantly between the two types of rice varieties (Sun et al., 1983) Varietal (cultivar) differences have also been reported in oats (Mc Coy et al., 1982), maize (Zehr et al., 1987), wheat (Mahmand et al., 1990) and Medicago sp. (Nagarajan and Walton, 1987). The reasons for such genotypic differences can be attributed to preexisting variation (Morrish et al., 1990) (such as polysomaty etc). There might be differences in the degree to which the tissue culture environment disrupts the cellular environment of a particular line (Peschke and Phillips, 1992).

Some cultivars may have genes that control tissue culture regeneration; others may not have the genes for regeneration or the genes controlling phytotohormone signals, but the trait can be transferred via traditional breeding methods into these genotype (Smith and Quesenberry, 1995). Genes on different chromosomes are involved in control of callus growth (Shimada and Makino, 1975; Baroncelli et al., 1978) and in the regeneration of shoots (Galiba et al., 1986;
Mathis and Fukui, 1986; Mathis et al., 1988; Kaleikau et al., 1989), suggesting a polygenic system (Henry et al., 1994) governing induction of cells involved in embryogenesis. Certain genes have a major effect on somatic embryogenesis and regeneration (Brown and Atanassov, 1985), whereas the lack of certain genes on other chromosomes may suppress embryogenesis (Henry et al., 1994). Genetic control of somatic embryogenesis in alfalfa (Medicago sativa L.) was found to be under the control of two dominant loci (Crea et al., 1995).

Embryogenic callus production, shoot regeneration and root regeneration are controlled by recessive genes and are inhibited by dominant suppressers. Production of non-embryogenic callus is determined by dominant genes that can give an additive effect. The lack of in vitro response is caused by at least two interacting genes acting in a suppressive fashion in rye.

Genetic analysis of regeneration ability has also revealed that the trait can be controlled by a few genes (one to three loci) with quantitative and highly heritable effects (Reish and Bingham, 1980; Charmet and Bernard, 1984; Brown and Atanassov, 1985; Mclean and Nowak, 1989; Nadolska-orcayk and Malepszy, 1989), polygenic control strongly influenced by environment (Tomes and Smith, 1985), polygenic control with little environmental influence (Rakoczy-Trojanowska and Malepszy, 1995), dominant genes (Komatsudo et al., 1989; Nadolska- Orczyk and Malepszy, 1989; Reish and Bingham 1980) having positive heterotic effects (Fleihinghaus et al., 1991).

(3) Culture age: Increase in somaclonal variations of all types have been reported with an increase in culture age (McCoy et al., 1982; Benzion and Phillips, 1988; Muller et al., 1990). The effect of age has been attributed to one or a combination of following causes by Peschke and Phillips (1992).

- Culture per se becomes more prone to change as it gets older, i.e., the mutation rate per cell generation increases.
- A number of mutations takes place early but detected only when a sufficient number of mutant cells have accumulated.
- Mutations occurring at an early culture age are actively selected.
- Increased polyploidy (Murashige and Nakano 1967; Colijn- Hooyamns et al.,
Benzion and Phillips (1988) analyzed pedigrees of a large number of callus lines and found that cytological aberrations detected in plants regenerated after many months in culture could often be traced back to a common subculture point early in the culture process. In contrast, Fukui (1983) reported that mutations detected in rice regenerants appeared to have occurred at various stages throughout the culture process.

Barbier and Dulieu (1983) using a genetically marked explant source, have shown that while most genetic changes occur in the first few mitoses in culture, some genetic changes increase with the duration of culture. Lorz and Scowcroft (1983) showed that by doubling the duration of culture, the frequency of genetic changes increased from 1.4 to 6% in protoplast of heterozygous clones.

It has been increasingly indicated by several authors that extended culture produces an elevated frequency of somaclones. Somatic hybrids are found to be a richer source of variability than sexual hybrids (Evans et al., 1982).

It has been reported that as callus tissue increases, the morphogenesis potential decreases, whereas, the frequency of albino shoots and only root producing callus increases (Wen et al., 1991). The highest frequency of chlorophyll variations found in regenerated plants from inflorescence cultures come from 210+ days old cultures (Cai et al., 1990).

The degree and extent of polyploidy in an in vitro culture tends to increase progressively with increasing age of the primary explant or callus or under particular hormonal regimes (Melchers and Bergman, 1958; Mitra and Steward, 1961; Blakely and Steward, 1964; Murashige and Nakano, 1967; Demoise and Partanen, 1969; Mehra and Mehra, 1974). An important mechanism of polyploidization in vitro appears to be restitution nucleus formation due to spindle failure and chromosome lagging at anaphase (Bayliss, 1973).

Among the various ploidy levels occurring in cultures of somatic plant tissues of special interest are odd-ploid chromosome numbers; haploid, triploid, pentaploid etc. Haploid and triploid mitoses are reported to occur in in vitro
cultures of *Happropappus gracilis* (Mitra and Steward, 1961; Shamina, 1966); a callus strain that showed 13% triploid mitoses (3x=6) at the fourth month of culture (Shamina, 1966), when examined several years later, was found to comprise a wide range of ploidy from diploidy (Sidorenka and Kunakh, 1970).

(4) **Culture conditions:** Growth regulator composition of the culture medium has been indicated to influence frequency of karyotypic alteration in cultured cells (Bayliss, 1975; Deambrogio and Dale, 1980).

**Hormonal factor:** Callus is initiated *in vitro* on cut or exposed cell surface in contact with a growth medium. Callus proliferation is a wound response. Excision of the explant stimulates the wound response *in vivo* which can be enhanced by growth regulators (McClintock, 1984). Most plant growth regulators, and specially, 2, 4-dichlorophenoxyacetic acid (2,4-D) and benzylaminopurine have been implicated in tissue culture induced variability (D'Amato, 1985; Evans, 1988; Grierback *et al.*, 1988; Shoemaker *et al.*, 1991; Skirvin *et al.*, 1994).

The primary event causing tissue culture-induced variability may be cell cycle disturbance (Peschke and Phillips, 1992) caused by exogenous hormone effects (Bayliss, 1977a, b; Bhaskaran and Smith, 1990; Liscum and Hangarter, 1991) or nucleotide pool imbalances (Jacky *et al.*, 1983). Auxins can produce rapid disorganised growth during callus induction that may lead to genetic instability through asynchronous cell division (Gould, 1984; Lee and Phillips, 1988). Increased thymidine can enhance chromosome breakage (Ronchi *et al.*, 1986; McClintock, 1978), chromosome breakage could lead to aneuploidy (*via* chromosome fragment loss), activation of transposable elements, methylation changes (Grafstrom *et al.*, 1984), initiation of SOS response resulting in single base changes (Walker, 1984), and an error-prone repair system (Burr and Burr, 1988). Exposure of plant cells to culture may provide conditions for disruption of genomic stability (due to the behaviour of repeated DNA sequences) leading to release of variability (Bhaskaran, 1987).

Kinetin (K) acts as a trigger for mitosis in endoreduplicated cells (Torrey, 1961; Van'T HOF and Macmilan 1969) and in cultured pea root cortex cells it can induce endoreduplication prior to mitosis (Libbenga and Torrey, 1973). Selective
induction and maintenance of mitosis in cells of different ploidy levels was first demonstrated in pea root segments, cultured in vitro on Shigemura's synthetic medium, the diploid cells only proliferated; when K (or yeast extract) plus 2, 4-D were added to the medium, the tetraploid cells were selectively stimulated to mitosis and the proliferating fraction of the culture consisted of tetraploid cells only (Torrey, 1961, 1967; Matthyse and Torrey, 1967). Direct as well as inverse correlation have been reported between polyplody in tissue cultures and the presence of 2, 4-D in the medium. Some authors regard it as a direct inducer of polyplody (Sunderland, 1977; Mitra and Steward, 1961), others considered it as a factor selectively favouring the divisions of polyplloid cells. An inverse relationship between the concentration of 2, 4-D and the degree of polyplloidization was reported in Pisum sativum (Kallak and Yarvekylg, 1971) and in Haplopappus gracilis and Vicia sp. (Singh and Harvey, 1975). Since a combination of auxin and cytokinin is essential for DNA synthesis and mitosis (Skoog and Miller, 1957) their quantitative ratios in a culture medium can greatly influence the composition of the proliferating cell population, as shown with H. gracilis callus. Bennici et al. (1971) noted that increasing concentration of NAA and kinetin decreased the frequency of polyplloid mitosis.

(5) Ploidy factor: Regenerated plant variability is higher among polyplloid and high - chromosome number explant sources (Heinz and Mee, 1969, 1971; Creissen and Karp, 1985) than among lower ploidy and low-chromosome number species. Spontaneous doubling is a common type of chromosome aberration in many diploid dicotyledonous regenerated plants (Murashige and Nakano, 1966, 1967) whereas tetraploids regenerated from callus or protoplasts often have chromosome structural changes, aneuploidy, and chromosome doubling (Bingham and McCoy, 1986). Polyplloid in tissue culture is generally the product of either endopolyplloidization or nuclear fusion (Sunderland, 1977; Bayliss, 1980). Aneuploid reinsertions are more common in polyplloid than in diploid of same species (Peschke and Phillips, 1992).

(6) Karyological Aberrations: Chromosome abnormalities of in vitro regenerated plants can include numerous karyological changes (Karp and Bright, 1985; Lorz et al., 1988). Chromosome breakage and its consequences (deletions,
duplications, inversions and translocations) cause common aberrational events (Sacristan, 1971). The breakage positions are not random, but involve late replicating chromosome regions characterized by heterochromatin (Lima-de-Faria, 1969; Sacristan, 1971; McCoy et al., 1982; Lapitan et al., 1984; Marata and Orton, 1984; Johnson et al., 1987; Lee and Phillips, 1987; Benzoin and Phillips, 1988). The role of heterochromatin in causing chromosome breakage may be due to later than normal chromosome replication in tissue culture, with the chromatids being held together and creating a stress that results in breakage between the centromere and late-replication region (McCoy et al., 1982; Lee and Phillips, 1988). Tissue culture has also resulted in increased frequencies of sister chromatid exchange (Dolezel and Novak, 1986; Dimitrov, 1987; Dolezel et al., 1987) and somatic crossing over involving two homologues instead of two chromatids (Lorz and Scowcroft, 1983). Somaclonal variation may not be random because specific loci may have higher mutation rates than others during the in vitro process (Xie et al., 1995).

**Positive attributes of somaclonal variation:**

- It is a cheaper technique as compared to somatic hybridization and transformation, DNA recombination.
- It results in a rich source of genetic variability.
- It is not necessary to have identified the genetic basis of the trait, as in the case of transformation where isolation and cloning is required.
- Novel variants have been reported among somaclones, and genetic (Compton and Veilleux, 1991) and cytogenetic evidence indicate that both the frequency and distribution of genetic recombination event can be altered by passage through tissue culture.
- Plant with genetic variability can be directly transferred to the field and evaluated as part of an ongoing programme.
- Somaclonal variation also hold promise as an adjunct to protoplast fusion (Evans and Sharp, 1986).

This suggests that variation may be generated from different areas of the genome than those that are accessible to conventional and mutation breeding. It is
not a precision tool and only minimal control can be exercised over its operation, and can be an important tool in crops with narrow genetic bases.

2.5. Chromosomal aberrations in cultured cells and regenerated plants

Variation in chromosome number and structure has been observed among cultured cells and regenerated plants as indicated by several workers (Bayliss, 1980; D’Amato, 1985; Lee and Phillips, 1988; Peschke and Phillips 1992). These variations can be grouped in following types:

1. Polyploidy/Aneuploidy - Variant plants with altered chromosome number have been reported by several authors. Polyploidy is the most frequently observed chromosomal abnormality and is generally explained as the product of either endopolyploidization or nuclear fusion (Sunderland, 1977; Bayliss, 1980). Aneuploidy may be caused by nondisjunction, lagging chromosomes, aberrant spindles and chromosome breakage producing dicentric and acentric chromosomes (Sunderland, 1977), or polyploidy followed by chromosome elimination (Balzan, 1978).

Aneuploidy is better tolerated in polyploid species than diploid ones, due to the greater imbalance of genetic material in the diploid situation. Changes in chromosome number are commonly associated with reduced fertility and with altered genetic ratios in progeny of self fertilized plants (Evans and Sharp 1986). Presence of polyploidy (up to 64 chromosomes instead of normal 2n=4), aneuploidy and anaphase abnormalities as well as morphologically altered chromosomes in cells of predominantly normal diploid Haplopappus culture has been reported (Mitra and Steward, 1961).

In culture, irregular polyploidy or aneuploidy may occur in different parts of the culture, in localized areas. This reflects that replication or multiplication of chromosomes may have preceded in different cells at different rates (Krikorian et al. 1983). In some cell lines of daylily plants, the majority of cells in each population is represented by a zone of connecting chromosome numbers or gradation in numbers (Krikorian et al., 1983).
D'Amato (1972) and Vant’of (1974) supported the view that proliferating or potentially proliferative cells in a species are genetically determined to arrest in a certain cycle phase; for example ‘G’ phase. It seems plausible that, in a given species, the cell cycle control which operates in vivo also operates in vitro. Leaf callus of *Crepis capillaris*, when analyzed cytologically and cyphotometically, was found to consist of diploid cells only up to one year of culture (Reinert and Kuster, 1966); but in the course of time, polyploidy began to appear and increased with time to reach 28% after 20 months (Sacristan, 1971).

In the majority of plant species, differentiated tissues in vivo, contain endopolyploid nuclei, that is nuclei whose chromosomes at interphase have undergone one to several duplications: up to twelve in the polytene chromosome cells of the suspensor of *Phaseolus caccineus* and *P. vulgaris* (Nagl, 1974). Polyploid and aneuploid regenerated plants or shoots have occurred in cell or tissue cultures of many species (D'Amato, 1977). Plants with doubled chromosome number were produced from over 100 sugarcane clones (Heinz et al., 1977). Nine octoploids were found among 200 regenerated alfalfa plants from tissue cultures initiated from tetraploid alfalfa (*Medicago sativa*) by Saunders and Bingham (1972). Genotypic effect was attributed to the observation in which regenerants of one variety of tobacco were diploid, where are those of another were tetraploid (Dulieu, 1972).

2. Chromosomal breakage: McCoy et al., (1982) proposed the hypothesis for role of heterochromatin in causing chromosome breakage due to its late replication. In addition, this could also result in nondisjunction and subsequent aneuploidy. Sacristan (1971) found that in *Crepis capillaris* callus culture, the SAT-chromosome was involved in 82% of the rearrangements, with the break point (s) corresponding to a region of late DNA synthesis. Chromosome breakage in regions of late DNA synthesis (likely heterochromatic) seems consistent with observations of tissue culture induced chromosome rearrangement in many cases (Armstrong et al., 1983; Ashmore and Gould, 1981; Lee and Phillips, 1987, 1988). In tissue culture of *Haplopappus gracilis* frequent occurrence ofacentric fragments, microchromosomes, deleted chromosomes, dicentric chromosomes and ring chromosomes have been noted (Singh 1975).
In cultures of somatic cells, simultaneous breakage of homologous chromosomes could lead to duplications and deficiencies whereas simultaneous breakage in nonhomologous chromosomes could lead to reciprocal interchanges (Lee and Phillips, 1987).

3. Nucleotide pool imbalance: The effect of nucleotide pool imbalance have been documented for a wide variety of species. Plant cells in tissue culture may be especially susceptible to dNTP (deoxyribonucleotide) pool imbalance because they can be serially transferred from depleted to fresh media almost indefinitely (Lee and Phillips, 1988).

4. Mitotic recombination: Various forms of mitotic recombination, including somatic crossing over and sister chromatid exchange (SCE) could produce several types of chromosomal rearrangements observed in tissue culture, especially if the exchange were asymmetric or between nonhomologous chromosomes (Larkin and Scowcroft, 1981). In Haploappus culture, chromosomal arrangements strongly resembling chiasmata was reported in somatic cells during mitosis which, may represent mitotic crossing over or segregation (Mitra and Steward, 1961). Dolezel et al., (1987) also reported that SCE frequency increase with a low concentration of 2, 4-D. Dimitrov (1987) observed that most of SCE is Crepis capillaris occurred at the junction between early and late replicating regions. Higher frequency of SCE was reported in callus in comparison to root tips (Dolezel and Novak, 1986).

5. Translocations: Translocations have been reported in cultured cell population of Crepis sp. (Sacristan, 1971), Carrot (Bayliss. 1975), Allium sativum (Novak, 1974, 1981).

2.6. Isozymic studies in somaclonal variants

In the broad sense, isozyme refers to any two distinguishable proteins that catalyze the same biochemical reaction. Isozymes are multiple molecular forms of an enzyme with similar or identical substrate specificities occurring within the same organism (Markert and Moller, 1959). This phenomenon came under extensive study after development of zymogram technique by Hunter and Markert
(1957). This technique involves electrophoretic separation of tissue extract, followed by demonstration of zones of enzyme activity using specific histochemical staining procedure applied directly to the electrophoretic medium. This technique allows for the resolution of isozymes mainly on the basis of charge and size differences. It does not discriminate mutants that may have similar electrophoretic mobilities, but may differ in physicochemical properties.

Isozymes are direct gene products and are, therefore, less susceptible to modification by environmental factors. Since enzymes are coded for by genes, any disruption in the coding sequence even at the single base level could force variations in the expression of the enzyme, leading to an altered individual. Variation detected in isozyme studies have genetical basis, thus they are used as markers in many biological studies. The regeneration of whole plants from cultured cells has been an area of intense investigation. Isozymes may provide a unique tool in such studies.

Isozyme patterns have been used to detect changes during regeneration as they play a vital role in development and differentiation (Orton, 1983, Chawla, 1988). Isozyme study has been suggested as a valuable tool in identifying genetic and epigenetic changes (Michel, 1975). Isozymes constitute ideal markers for tissue and somatic cell genetic studies due to:

- The ease of detection
- The abundance of naturally occurring variants in most plant populations.
- Applicability to small amounts of tissue and crude extracts.
- The marker is mostly expressed in the “undifferentiated” state of a cell culture.
- Many loci express at all stages of the development.
- Genetic inheritance can be easily demonstrated. Most loci have Mendelian inheritance.

Isozymes are used primarily in plant tissue culture for physiological studies. Peroxidases in *Nicotiana* (Lee, 1971; 1972) and in *Pelargonium* culture (Lavee and Galston, 1968) were reported to vary in cultures. Definite qualitative changes in the isozyme patterns of peroxidase, esterase and acid phosphatase were observed in response to varying light and temperature conditions (McCown *et al.*, 1968).
Various authors have reported, isozymic variation during tissue culture. Variation in esterase and acid phosphatase was reported in tobacco callus culture (Bassiri and Carlson, 1979), in wheat and barley calli during differentiation (Chawla, 1988). Novel acid phosphatase bands were observed during cytodifferentiation in callus cultures of *Vigna* (De and Roy, 1984). Isozyme studies as markers to distinguish between embryogenic and non-embryogenic calli in *Panicum maximum* was suggested by Alarmelu *et al.* (1999). They found acid phosphatase to be organ and tissue specific. Isozyme pattern of esterases were found to vary with developmental stages. Twelve isoesterases were detected in the embryogenic calli, out of which only two are retained at the time of germination of embryoids into plantlets.

Study of different isozymes such as acid phosphatase in *Panicum maximum* (Alarmelu *et al.*, 1999), in other grasses (Lorenc-kubis and Marawiecka, 1985); esterases in guinea grass (Alarmelu *et al.*, 1999), in maize (Everett *et al.*, 1985) has been suggested for differentiating between embryogenic and non-embryogenic calli.

Variation in glutamate dehydrogenase was reported at various stages of callus growth in *Phaseolus vulgaris* (Arnison and Boll, 1974). They reported change from a pattern of five to a single electrophoretic band after subculture and gradual return to five at end of the culture period.

In hexaploid wheat, study of 17 somaclonal variants show chromosomal changes as well as change in ADH-1 phenotype (Davies *et al.*, 1986). Alteration in phosphoglucomutase and shikimate dehydrogenase phenotype were observed in regenerated celery plants, which were also correlated with karyotypic changes (Orton, 1983).

No variation was detected in protoplast derived Russet Burbank potatoes in a screening involving 13 enzyme systems (Sanford *et al.*, 1984). Similarly 180 regenerated garden pea plants showed same zymograms for esterase, glutemate dehydrogenase, 6-phosphogluconate dehydrogenase and leucine amino peptidase.
(Rubluo et al., 1984). Study of 14 isozyme systems in 63 plants of napier grass (*Pennisetum purpureum*) did not show qualitative variation on any Loci (Shenoy and Vasil, 1992).

Isozyme analysis can also explain the genetic basis of certain somaclonal variations. If the variation is caused by a loss of a large portion of a chromosome or it involves the induction or removal of a large number of genes, it is expected that it will result in loss or addition of isozyme bands relative to standard phenotype. In contrast, if only a single gene is altered, it may not reflect in isozyme pattern.

Changes in isozyme pattern in somaclonal variants has been reported in celery (Orton, 1983) and wheat (Davies et al., 1986). Modification of wheat B-amylase phenotype with additional activity bands, where the meiotic and mitotic chromosomal configurations were normal have been reported by Ryan and Scowcroft (1987).

2.7. *In vitro* regeneration studies in *Trifolium* species

*Trifolium*, being an important genus from fodder and pasture point of view, has attracted attention of various research workers for its genetic improvement through biotechnological tools. Many researchers have worked on important temperate *Trifolium* species such as *T. repens*, *T. pratense*, *T. subterraneum* etc. Sporadic efforts carried out through 1970s and 1980s got a boost after classical works of Phillips and Collins who devised a new basal media L2 for *T. pratense*. Only few reports are available on tropical and sub-tropical species such as *T. alexandrinum* and *T. resupinatum*. A brief account of *in vitro* organogenesis and embryogenesis studies carried out in different species are given below:

*T. alexandrinum* (Egyptian clover or Berseem clover):

Plant were regenerated from hypocotyl and anther explants of berseem clover on MS medium containing various combination of plant growth regulators.

Mokhtarzadeh and Constantin (1978) cultured seedling hypocotyl section of berseem clover on MS medium containing various combinations of IAA, NAA,
2, 4-D, 2-ip, BA and KIN. Optimal callus induction occurred on a combination of 5.5 μM NAA and 7.5 μM KIN. Berseem clover was very sensitive to 2, 4-D. Callus was subsequently propagated on medium containing 11 μM NAA and 0.45 μM 2-ip. Cell suspension cultures were grown from callus inoculated in liquid medium containing 11 μM NAA and 0.9 μM 2-ip. Callus colonies were recorded from all suspensions inoculated on a solidified version of the same medium. Immature anthers excised from one plant also formed callus. Callus of all sources produced shoots when cultured on medium containing 2.7 μM NAA and 2.5 μM KIN. Certain other combinations of growth regulators yielded shoots less efficiently. Shoots were rooted on medium containing 5.7 μM IAA and 0.44 μM BA. Plants were established in the green house.

Induction of shoots on MS medium with 0.5 mg/l each of NAA and KIN followed by induction of roots on MS medium with 1.0 mg/l of 6-BAP- suspension cultures in liquid. MS medium containing 2 mg/l of NAA and 0.2 mg/l of 2-ip provided filterable cell preparation with 45% viable cells. 4% of which gave rise to colonies within 3 weeks of transfer to agar plates. Shoot development was observed when callus from the colonies was cultured on MS medium with 0.5 mg/l of NAA and KIN. Twenty per cent of uncontaminated anthers from a single plant cultured on MS medium containing 1.0, 0.1 and 0.01 mg/l of NAA, 2, 4-D and 2-ip, respectively produced callus which could be maintained and from which plants could be regenerated by culturing on MS medium with 0.5 mg/l each of NAA and KIN. Preliminary results indicate that cells of root tips from hypocotyle and anther derived callus have the expected diploid and haploid number of chromosomes (2n=16).

Barakat (1990) screened a range of MS based media for their ability to induce callus from root, hypocotyl and cotyledon explants of four T. alexandrinum cultivars. The MS medium containing 2.0 mg/l NAA and 0.5 mg/l BAP (MSP-1) was the one which gave optimum callus (Friable and fast growing) induction for all sources of tissues. However, callus growth was minimal on MS medium containing 0.05 mg/l NAA and 0.5 mg/l BAP (MSD-4).
MS medium containing 2.0 mg/l NAA and 0.5 mg/l BAP was highly effective in producing green nodules from the different callus types of all cultivars. Shoot regeneration was observed after 2 subculture on same media (MSP-1). Root formation was induced when regenerated shoot tips (1.2 cm) were transferred to MS based agar medium with 2.0 mg/L NAA, 0.03 mg/L Kinetin and 0.001 mg/L folic acid. Variation between cultivars has been observed mainly as effects of genotype on the regeneration of plants from callus (Barakat, 1990).

**T. medium (Zigzag clover)**: Petiole segments from two strains (CZO and Beaver lodge) of zigzag clover (*T. medium*) were cultured on L2 or in SL2 medium. Shoots were regenerated via organogenesis as well as somatic embryogenesis from petiole segments of both strains. Direct shoot regeneration was noticed as early as eight days after the initiation of cultures. Regenerated plants have normal morphological characteristics (Choo, 1988). In an earlier study, no regeneration was obtained from callus culture (Parrott and Collins, 1982).

**T. pratense (Red clover)**: Initial attempts to generate callus from red clover tissue and plants from callus were disappointing. Niizeki and Kita (1973) cultured 99 anthers from two cultivars on the basal media of Miller (1961) and Bourgin and Nitsch (1967) modified with various combinations of IAA, NAA, 2, 4-D, BA and GA. Callus formation occurred only in a single instance on Miller medium containing 17 μM IAA and 6.6 μM BA. However, no morphogenetic development was observed. Microscopic evaluation indicated that the callus arose from somatic tissue.

Ranga Rao (1976) initiated callus from root, stem and leaf tissues of one cultivar and two breeding lines of red clover cultured on a basal medium modified from Miller (1961) and supplemented with 9 μM 2, 4-D and 10 μM KIN. Callus suspensions were subsequently cultured on liquid medium free of NO₃⁻, 2,4-D and KIN and inoculated with a symbiotic strain of *Rhizobium trifolii*. In 6-8 weeks, 75% of the infected cultures produced roots regardless of the origin of the tissue. Un-inoculated tissues did not undergo morphogenesis. It was concluded that hormones produced and released by the *Rhizobium* in infected cultures were
responsible for root formation by the red clover callus.

Various combinations of 2, 4-D, IAA, NAA, KIN and corn milk were evaluated using a basal medium composed of the major salts of Gautheret (1955), minor salts modified from Burkholder and Nickell (1949) and organic components modified from Gautheret (1955) callus growth was obtained using 9 mM 2, 4-D and 15% corn milk. Very little growth occurred without the addition of corn milk. This suggests that undefined nutritional factors were missing from the basal medium (Zakrzewski and Zakrzewski, 1976).

Ahloowalia (1976) cultured seeds of one tetraploid cultivar on MS medium supplemented with 7.5 μM 2, 4-D, 37 μM IAA, and 10 μM KIN. Callus was produced from 63% of the seeds within 6 weeks. Somatic embryos at the globular, heart, and torpedo stages were recovered from callus cultured on half strength MS medium free of growth regulators. No plants were regenerated.

Phillips and Collins (1979) investigated the growth of callus from seedling of five cultivars. Initial experiments evaluating the SH, B5 and MS basal media containing 2, 4-D and KIN were not very productive. Visual ratings of various combinations of NAA, IAA, CPA, 2, 4-D, PIC, KIN, 2-ip and BA were carried out. The combination of 0.25 μM PIC and 0.44 μM BA was optimal for callus initiation and cell proliferation. Since other basal media were deemed unsatisfactory, an improved basal medium designated L2 was experimentally developed (Phillips and Collins 1979). The chemical forms and concentrations of individual components were visually rated. Major and minor inorganic elements and organic compounds were evaluated in that order. The final composition of the L2 medium contained a major salts formulation similar to that of the MS medium, with a reduction in the concentration of NH4+ and increased concentrations of PO4−, K+, Mg++ and Ca++. The minor salts formulation was similar to that of the SH media with adjustments in the concentration of several salts. The organic formulation (Vitamins and Sucrose) was similar to that of Linsmaier and Skoog (1965) media with increased concentration of thiamine and myo-inositol. The addition of nicotinic acid was inhibitory to the callus growth. The L2 medium was found to be more broadly supportive of red clover genotypes in culture than other
basal media. In tests with alfalfa (*Medicago sativa* L.) and soybean (*Glycine max* (L.) Merr.) the L₂ inorganic formulation was statistically shown to be superior to the MS, SH and Miller formulations, confirming that the L₂ medium was more broadly supportive of certain legumes in culture. The frequency of regeneration was dependent on the genotype and explant, with callus tissues derived from meristem having a higher regeneration capability than those from non meristematic regions (Phillips and Collins, 1979).

Phillips and Collins (1979) obtained vigorous callus from both mature and immature vegetative and reproductive explant sources on the L₂ medium. Plants were regenerated from callus cultures. The frequency of plant regeneration was depended on the origin of the tissue and the source cultivars.

Cheyne and Dale (1980) obtained regeneration from shoot tip explant of one diploid cultivars and one tetraploid cultivar. B5 or Blaydes basal media supplemented with 1.2 μM IAA and 0.9 μM 2-ip yielded about 67% plant regeneration frequencies. Bhojwani et al., (1984) could not obtain organogenesis on MS supplemented with 2,4 D or BAP or 2-ip in various combinations with or without CH.

*T. subterraneum* (Subterranean clover):

Earlier study of Graham (1968) failed to get any organogenesis. He cultured seeds of subterranean clover on modified MS medium (Linsmaier and Skoog, 1965, Supplemented with 4 μM nicotinic acid) containing 0.2 μM KIN and 1 μM 2, 4-D to induce callus formation. Callus was maintained on the same medium. Cell suspension cultures were grown on a liquid version of the same medium. Cultures were inoculated with *Rhizobium* which did not interfere with growth. No morphogenetic development was observed.

Regeneration of *T. subterraneum* was achieved by both shoot organogenesis and somatic embryogenesis. Regeneration in subterranean clover was obtained by culturing seedling hypocotyl on L₂ medium. Shoot bud development was clearly visible on hypocotyl explants after three to ten days in L₂ medium (i) shoot with trifoliate leaves were than formed often one month in culture. These shoots
developed from the hypocotyl region whereas the radicle region showed no further development. Histological studies revealed that shoots arose de novo and did not originate from pre-existing meristems. Shoot derived via organogenesis produced roots within two weeks of transfer to RL medium and the regenerated plants had a normal phenotypic appearance. Hypocotyl explants showed no shoot but development in the absence of added growth regulators (L2 medium) (Heath et al., 1993).

In the second regeneration protocol, Shoot apices taken from 10 days old seedlings of the cultivar Dalkeith were induced to form calli on L2 basal medium supplemented with the auxin picloram (21 μM) and maltose (4%) as the sugar source. Somatic embryos appeared spontaneously on the calli. A majority of embryos had a well-defined root pole, two cotyledon and were capable of germination, albeit at a low frequency. Regenerated plants obtained from both protocols appeared phenotypically normal.

*T. repens* (White clover):

Pelletier and Pelletier (1971) cultured cotyledons of one white clover cultivar. Callus was grown on a medium composed of MS major salts, Heller minor salts, B vitamins, agar, 0.17 M glucose, 2,4-D and KIN. Callus was transferred to medium containing 0.5 μM NAA, 0.5 μM KIN and 10% CW after 6 weeks. Callus derived from a single cotyledon exhibited organization. They both chromosomal and morphological variation in the regenerates. However, in later work, Gresshoff (1980) was unable to find any chromosomal differences in morphologically variant regenerants, even in plants obtained up to 2 years after culture initiation.

Rupert and Seo (1977) regenerated plants from callus derived from embryos following sexual hybridization of white clover with *T. ambigum*, the callus remaining totipotent for up to 3 years in culture. Maheswari and Williams (1984) cultured immature embryos of white clover, and obtained a clone of axenic plantlets through primary and secondary embryogenesis.
Bhojwani *et al.* (1984) and White (1983, 1984) emphasized the importance of selection for regenerating genotypes in white clover, and isolated two plants, TR-20 and WR8, with high regeneration capacity. Bhojwani *et al.*, (1984) could obtain plantlets on MS media supplemented with 2-ip (0.5 mg/L) and IAA (0.1 mg/L).

Parrott and Collins (1982) have successfully obtained callus and cell suspension cultures from white clover using the methods and media developed by Phillips and Collins (1979, 1980) for red clover. Callus derived from 20% of the tested genotypes produced roots.

Gresshoff (1980) cultured seeds and seedling section of one cultivar on B5 or MS basal media supplemented with 0.5 μM KIN and either 4 or 10 μM 2,4-D. Callus induction was complete on the higher concentration of 2, 4-D.

Richard and Rupert (1980) found that a modified MS medium containing 2.9 M IAA and 4.5 M 2-ip encouraged shoot multiplication from cultured embryos.

When primary somatic embryos derived from immature sexual embryos of *T. repens* are sub cultured in a medium containing 1-2 mg/l BAP. Secondary embryos arise directly by multicellular proliferation of superficial cells on the lower surface of the axis and cotyledons. The secondary embryos develop into small leafy shoots on the induction media and are easily detached for rooting on hormone free medium. Specialization of walls determining embryos from tissue apparently contributes to this case of separation. Using this secondary subculture technique, it is possible to obtain a clone in the order of 100 rooted plantlets from one immature sexual embryos within 10-14 weeks from pollination. (Maheswaran and Williams, 1986)

Cotyledons from immature embryos of *T. repens* var. Oscola were exposed to 2, 4-D or NAA to induce somatic embryogenesis. NAA at 10 or 20 mg/l was very inefficient at stimulating embryogenesis, while concentrations of 30 or 40 mg/l resulted in death of the explant tissue. Continuous exposure of cotyledons to 40 mg/l, 2, 4-D resulted in somatic embryos which are arrested at the globular stage. A 10 day exposure time to 2, 4-D at the same concentration led to formation
of somatic embryos, most of which had poorly developed cotyledons. Almost 10% of the somatic embryos converted into plants following transfer to medium devoid of growth regulators. Attempts to improve morphology of somatic embryos by using shorter exposure times to 2, 4-D at 40 mg/l or by maintaining the 10 day exposure time while varying the concentration of 2, 4-D were not successful. (Parrott, 1991)

Somatic embryos were obtained from immature cotyledons of white clover cultivar Oscola placed into EC6 basal medium containing 40 mg/l of 2, 4-D and 6% sucrose. Repeated sub-culture of white clover somatic embryos on EC6 basal medium containing 6% sucrose with 2,4-D at 20 or 40 mg/l effectively maintains repetitive embryogenesis. Medium containing MS salts with 6% maltose as the carbohydrate source was the most efficient for plant recovery. (Weissinger and Parrott, 1993).

In white clover most prolific and rapid plant regeneration occurred on MS based media containing NAA and BAP, while other phytohormone combinations, 2, 4-D or picloram with kinetin 2-ip resulted in either extensive callus formation or distorted shoot development (White and Voisey, 1994). They conducted a series of experiments to screen the potential of roots, hypocotyl and cotyledons from seedlings to regenerate. Results indicated that cotyledon from 3 day old seedlings were most responsive. Regeneration was obtained from the cotyledon of white clover using MS basal medium supplemented with varying concentrations of NAA and BAP. The highest shoot regeneration frequency (an average of 20 shoots per cotyledon) was obtained using MS medium containing 1.0 mg/l. BAP and 0.05 mg/l NAA. A similar regeneration frequency was obtained from cotyledon explants taken from eight different white clover cultivars and no genotypic effect was observed.

Other Clovers

Trifoliums of less economic importance than red and white clovers have also been cultured, and regeneration described for *T. incarnatum* (Crimson clover; Beach and Smith, 1979). Less attention has been given to wild species, although Parrott and Collins (1982) reported somatic embryogenesis from seedling explants

Schenk and Hildebrandt (1972) obtained callus growth from seedlings of Alsike clover cultured on SH medium containing 2.2 μM 2, 4-D+ 11 μM CPA and 0.5 μM KIN. No morphogenetic development was reported. Beach and Smith (1979) obtained callus from Crimson clover in the same manner as from red clover.

Parrott and Collins (1982) cultured large hop clover (*T. campestre*) subterranean clover, crimson clover, *T. alpestre*, zigzag clover and *T. rubens* following procedures developed by Phillips and Collins (1979, 1980) and Collins and Phillips (1982) for red clover. Large hop clover failed to respond to shoot tip culture while rapid clonal propagation was achieved by with *T. alpestre*, Crimson clover, *T. rubens* and subterranean clover. The latter species multiplied most rapidly in shoot tip culture. Zigzag clover produced callus as well as shoots on the standard medium which was correlated by using one third the normal concentration of PIC as auxin. Subterranean clover rooted prolifically on the standard rooting medium. Rooting frequencies of the other species were more efficient using only water. Callus of subterranean clover, large hop clover, and *T. alpestre* did not grow well, exhibiting varying degrees of necrosis. The L2 medium was suboptimal for these species. About 20% of the *T. alpestre* genotypes rooted before the callus turned brown. All callus of subterranean clover rooted. One callus sector of large hop clover survived and proliferated slowly. Zigzag clover and *T. rubens* produced callus which grow more vigorously than that of red clover, while callus of crimson clover grow more slowly. Some crimson clover callus developed buds and roots.