Chapter- II

Review of Literature

2.1. General Description

Banana and plantain develops from an underground stem called the 'corm' or 'rhizome'. The rhizome is a stout, tuberous and storage organ which sustains the growth of the bunch and sucker. It has extremely short internodes covered by closely packed leaf scars. The roots emerge from the rhizome, most of which grow horizontally to a depth of 15 – 20 cm in the soil (Champion and Siossaram, 1970). They are whitish and healthy when newly formed, but turn grey or brown as they age. The position and distribution of roots is influenced by type of soil, compaction and drainage. The uptake of essential minerals from the soil depends on the size and effectiveness of the root system (Tezenas du Montcel, 1987). Suckers are formed less than nine months after planting, depending on the genotype. However, most hybrids can develop suckers earlier.

Depending on the height and morphology of leaves, suckers can be differentiated into peepers, sword, maiden and water suckers. The water suckers have broad leaves and a narrow rhizome base. Contrarily the sword suckers have narrow leaves and are ideal for planting. The mother plant with the suckers constitutes a mat or stool. After the crop has been harvested, the mother plant is cut down and the suckers thinner. The second harvest from the plantain is the first ratoon crop. The third is the second ratoon and so on. Most plantain show strong apical dominance which retards ratoon growth and leads to mutual competition between the suckers once the infructescence on the main pseudostem is harvested (Swennen and De Langhe, 1985).

The pseudostem is a cylindrical structure, originating from the rhizome and containing about 95% water and it bears the leaves. Like in most monocots, the pseudostem consists of a system of concentric, overlapping leaf bases. Plantain produces about 35 leaves under normal conditions (Brouhns, 1957), depending on the cultivar. The scale leaves are first produced from the central meristem as the suckers

develop. The leaf consists of a sheath, petiole and lamina. Leaf size and rate of leaf emission depend on edaphic condition, climate and clone (Tezenas du Montcel, 1987). When the crop has formed a number of leaves, the terminal bud of the corm develops from the pseudostem and produces the inflorescence. At this stage, leaf production ceases. The inflorescence emerges between the leaves and is held in position by the peduncle. The inflorescence comprises the male, female and hermaphrodite flowers. Ovaries of the female flower develop into edible parthenocarpic fruits.

2.2. Somatic embryogenesis

Formation of an embryo from a cell other than a gamete or the direct product of gamete is known as somatic embryogenesis (Merkle *et al.,* 1995). It is not an artificial phenomenon restricted to *in vitro* work, but also occurs in nature, known as apomixis. In such conditions embryos develop from seed cells and tissues such as nucellus, integument, synergids, antipodals and endosperm, or through budding (cleavage polyembryony) from suspensor cells or apical cells in the embryonic mass produced by zygote (Sharma and Thorpe, 1995). Normally, tissues inoculated *in vitro* have to be subjected to plant growth regulators and other stress factors in order to induce somatic embryogenesis.

Pioneers of *in vitro* somatic embryogenesis were Wiggan (1954) and Steward *et al*. (1958), describing bud formation and proembryo like forms in *Daucus*. They were not able to clearly observe SE, but rather organogenesis and filamentous proembryos, and the first to demonstrate totipotency in somatic plant cells. In the early 1960s, reports appeared on the formation of bipolar embryos and plant regeneration there from in cultures derived from mature organs of the carrot plant (Steward *et al.,* 1963) or from cultured zygotic embryos (Norstog, 1961; Maheswari and Baldev, 1961).

Halperin (1967) introduced the term proembryogenic masses (PEMs) to describe the embryogenic cell masses from which somatic embryos can be formed. PEMs undergo cycles of growth and fragmentation until auxin is removed from the culture medium or drops below a threshold level in old cultures.

2.2.1. Phenotypic plasticity in plants

Plants are sessile organisms. In order to survive, grow and reproduce they have developed strategies to circumvent environment extremes and to take maximum advantage when conditions improve (Trewavas, 1981; Smith, 1990). This phenomenon, called phenotypic plasticity, includes instability in morphogenic and developmental programs and ability to respond to damaging external conditions. Concepts of competence, induction and determination, introduced by animal biologist, were taken over by botanists. Competence is exhibited if a cell/tissues/organ is exposed to a signal and responds in the expected manner. Prior to becoming competent, cells have to dedifferentiate. Induction refers to the expression as a unique developmental response from competent cells due to that signal. Determination is shown if an induced cell or group of cells exhibit the same developmental fate whether grown *in situ*, in isolation or at a new place in or on the organism. Due to plant plasticity, determination in plant cells is not as rigidly fixed as in animal cells.

2.2.2. Morphogenic competence and morphological expression

SE can be considered as an extreme case of phenotypic plasticity, where various states of development can be altered under appropriate conditions (Dudits *et al.,* 1995; Yeung, 1995). Altering the growth conditions and subjecting inoculated tissues and/or organs to unuasal conditions (stress), can trigger plant to abolish or alter gene expression related to a specific function they had in the plant and to become competent to the inductive signals for somatic embryogenesis. Another characteristic of plant cells is totipotency. All somatic cells within a plant contain the entire set of genetic information to regenerate into a complete and functional plant (Merkle *et al.,* 1995).

The three events (competence, induction and determination) enumerated above are usually tightly coupled and may be impossible to separate (Yeung, 1995). Hilbert *et al.,* (1992) reported on two critical phases involved in morphological differentiation of somatic cells into whole plants, namely induction of cells for morphogenic competance and morphological expression of that competence. Cells that are embryogenic are more easily induced to undergo somatic embryogenesis. Such cells, were called pre-embryogenic determined cells (PEDCs) (Evans *et al.,* 1981; Sharp *et al.,* 1982). Growth regulators or favourable conditions are then simply ‘permissive’ for cell division and expression of embryogenesis. In contrast to direct embryogenesis from

PEDCs, highly differentiated vegetative cells require major epigenetic changes. Growth regulators and or other stress factors are required to induce dedifferentiation, cell division and to determine the embryogenic state. These cells are called induced embryogenic determined cells (IEDCs). Induction of SE from these cells is indirect because an intermediate callusing phase is involved. The directness of embryogenesis therefore depends on the epigenetic 'distance' of explant cells from the embryogenic state (Merkle *et al.,* 1995). Once embryogenic determined cells are obtained, there appears to be no fundamental difference between direct and indirect SE (Williams and Maheswaran, 1986).

2.2.3. Unicellular and multicellular origin of somatic embryos

The question of a single- or multiple-cell origin for somatic embryoids is directly related to coordinated behaviour of neighbouring cells as a morphogenetic group. In agreement with Street and Withers (1974), Haccius, (1978) defined a non-zygotic embryo as a new individual arising from a single cell and having no vascular connection with maternal tissues. However, Raghavan, (1976) was more cautious in recognizing that a single cell origin had not been unequivocally demonstrated in many cases where apparently normal bipolar embryoids were formed cell from aggregates.

In both process, embryoids may arise from single cells (unicellular origin) or from a group of cells (multicellular origin), depending on whether or not neighbouring cells have the same interval development state and/or can communicate and behave as a group rather than as individual cells (Williams and Maheswaran, 1986). Unicellular or multicellular origin of the somatic embryos, is strictly related to the cell maturity or epigenetic distance from the embryogenic state in multicellular explants. Maheswaran and Williams (1985) illustrated this for induction of SE from epidermal cells. In their opinion, embryogenesis from a mature tissue is only possible by callus induction. In *Cichorium* however, direct embryogenesis was obtained from matured cortical cells in the root and from mesophyll cells in the leaf (Dubois *et al.,* 1990; Verdus *et al.,* 1993). Direct embryogenesis was also observed in mesophyll cells of Orchard grass (Conger *et al.,* 1983) and *Dactylis glomerata* (Trigiano *et al.,* 1987).

2.2.4. Application of somatic embryogenesis

2.2.4.1. Important applications

One of the main uses of somatic embryogenesis constitutes its employment as an approach to investigate the initial events of zygotic embryogenesis in higher plants. The mass propagation of plants through multiplication of embryogenic propagules is the most commercially attractive application of somatic embryogenesis (Merkle *et al.*, 1995).

Somatic embryogenesis over organogenesis:

a) It is the best method to culture large numbers of 'reproductive units' (60,000 to 1.35 million somatic embryos per liter of medium) with the presence of both root and shoot meristems in the same element.

b) This mode of culture permits easy scale-up transfers with low labor inputs since embryos can be grown individually and freely floating in liquid medium.

c) Pure culture of homogenous material can be obtained through somatic embryogenesis since they frequently originate from single cells.

d) Plants derived from somatic embryos are less variable than those derived by way of organogenesis (Ammirato *et al*, 1987; Merkle *et al.,* 1995; Osuga and Komamine, 1994). This is due to the intolerance of somatic embryos to mutations in any of the numerous genes that might be necessary for a successful completion of ontogeny (Ozias-Akins and Vasil, 1988), while vegetative meristems may be more tolerant to mutations and epigenetic changes (Merkle *et al.,* 1995).

2.2.4.2. Other applications

a) Somatic embryogenesis has opened up the possibility for the use of somatic embryos in synthetic seed technology (Gray, 1995).

b) Production of plants with different ploidy levels is another application of somatic embryogenesis *i.e*., obtaining haploid embryos by cultivating anthers and raising triploids from endosperm have been suggested and exploited to a very limited extent, exploited (Terzi and Lo Schiavo, 1990).

c) Embryogenic callus, cell suspension as well as somatic embryos can be used as a source of protoplasts, taking advantage of the totipotency (Merkle *et al.,* 1995).

d) Secondary or recurrent embryogenesis, which is reported in atleast 80 species (reviewed by Raemakers *et al.,* 1995), offers a great potential for *in vitro* production of embryo metabolites, such as lipids and seed storage proteins.

e) The embryogenic development of somatic cells appears to be more sensitive to the application of exogenous chemical compounds than the growth of whole plants or even callus cultures. This offers the possibility of using *in vitro* screening and selection procedures to identify plant genotypes resistant to certain factors, such as aluminum toxicity or toxins produced by pathogens (Merkle *et al.,* 1995).

2.3. Factors affecting somatic embyrogenesis

2.3.1. Effect of genotype

All plant cells contain the entire set of genetic information necessary to create a whole plant and, if proper induction conditions are provided, they can dedifferentiate into plants (Merkle *et al.,* 1995). Somatic embryogenesis capacity was found to change with genotype and their responses between genotypes have been studied in some plants such as alfalfa (Brown and Atanassov, 1986) and soybean (Parrott *et al.,* 1989). Genetic analysis suggested that this phenomenon is heritable and that one or more genes control the regeneration response. These genes belong to both nuclear and cytoplasmic genomes (McKersie and Brown, 1996). In nuclear genomes, the genes responsible for regeneration range from 1 to 2 in most tested plants (Henry *et al*, 1994). In both mono and dicotyledonous plants, the gene action of the nuclear genome is characterized as additive in regeneration and the regeneration-controlling genes vary among the different stages of the regeneration process. For instance, callus induction and somatic embryogenesis are controlled separately by one dominant gene and two complementary dominant genes in alfalfa (Hemandez-Femandez and Christie, 1989). These genes can be either recessive such as in wheat (De Buyser *et al.,* 1992) or dominant as in alfalfa (Reisch and Bingham, 1980). It appears that only a few genes control regeneration ability. In plants, the cytoplasmic genome consists of genetic material in mitochondria and plastids. They exist and function uniquely in cells, but are partially controlled by the nuclear genome and it has been reported that these organelles take part in callus production and affect regeneration capacity (Henry *et al.,* 1994).

2.3.2. Effect of explant

Somatic embryogenesis has been achieved from explants such as leaves (dos Santos *et al.,* 1980; Mezentsev and Karelina, 1982), petioles (Walker *et al.,* 1978, 1979), stems (Walker *et al*., 1978, 1979), cotyledons (Atanassov and Brown, 1984; Stavarek *et al.,* 1980) hypocotyls (Reisch and Bingham, 1980*),* protoplasts (Atanassov and Brown, 1984), microspores (Takahata *et al.,* 1991), tuber (Krishnaraj and Vasil, 1995), ovary (Brown *et al.,* 1995), ovule (Dunçtan *et al.,* 1995), nucellus (Dunstan *et al.,* 1995) and immature embryos (Walker and Sato, 1981; Walker *et al.,* 1978 and 1979). This is supported by the fact that though all organs, tissues and cells of plant body contain the same genetic information, but the reaction is different to *in vitro* tissue culture because of variation in their physiological state such as age (Takahata *et al.,* 1991) and degree of differentiation (McKersie and Brown, 1996). In monocots, cells differentiate early and rapidly followed by loss of mitotic and morphogenetic ability and explant source is considered even more important than genotype (Krishnaraj and Vasil, 1995). Young and undifferentiated cells are easier to regenerate than mature and differentiated cells (Duntan *et al.,* 1995; Krishnaraj and Vasil, 1995). Besides the degree of differentiation, levels of endogenous hormones differ among organs, tissues and cells, and some other unidentified factors may also determine the variation of regenerating responses (Brown *et al.,* 1995; Krishnaraj and Vasil, 1995). It is noteworthy that embryogenic cells of explants that respond to culture environment vary along with the environmental factors (Wenzel and Brown, 199l), possibly because of their different requirements for competence such as exogenous growth regulators (McKersie and Brown, 1996). The difference in regenerating ability among various organs, tissues and cells of a plant suggests that somatic embryogenesis is not only controlled by regenerating genes, but to a certain degree, also controlled by other factors such as physiological state of explants and culture conditions.

2.3.3. Effect of medium

The history of research in somatic embryogenesis can be described as a success story of medium development (Brown *et al.,* 1995). Success in somatic embryogenesis from a genotype or explant of a plant usually depends on components of the medium either directly or indirectly. MS medium (Murashige and Skoog, 1962) is among media which have been most widely used in somatic embryogenesis (Brown *et al.,* 1995; Dunstan *et al.,* 1995). More than 2,000 medium formulations have been reported, but

half of them are modified MS medium (Brown *et al.,* 1995). Other popular media include B5 (Gamborg *et al.,* 1968), SH (Schenk and Hildebrant, 1972), N6 (Chu *et al.,* 1975), and Linsmaier and Skoog, 1975) media (Brown *et al.,* 1995; Krishnaraj and Vasil, 1995). The culture media vary in both type and concentration of the components, but all media have similar basic classes of components which consist of growth regulators, nitrogen, carbohydrates, inorganic macro and micronutrients, vitarnins, and organic additives (Brown *et al.,* 1995). The interaction between genotype and medium components can greatly influence somatic embryogenesis. Among them, carbohydrates, growth regulators, nitrogen and growth regulators play the most important roles.

2.3.3.1. Carbon source

Sugar is carbon and energy source and it also takes part in osmotic adjustment (Denchev *et al.,* 1993). Its effects on somatic embryogenesis are dependent on the type and concentration of sugar, and the culture protocol. Through comparison and selection, it is now established that sucrose is the most suitable carbohydrate and widely used, followed by glucose, maltose, raffinose, fructose, mannitol and some other rarely used sugars (Denchev *et al.,* 1993; Lippmann and Lippmann, 1993). In some protocols, maltose is applied in medium for embryo development because maltose promoted more normal morphology than sucrose (Denchev *et al.,* 1993). The optimal concentration of sugar varies among sugar types, plant species and development stage of embryos. In terms of sucrose, optimal concentrations range from 58-88 μM (Brown *et al.,* 1999*)* or 2-4% for rapid growth of callus (George, 2006).

2.3.3.2. Nitrogen source

Nitrogen is indispensable to plant life and to somatic embryogenesis since it is required for the key components of plants namely structure and function and for materials such as proteins, nucleic acids, and plant hormones. The growth and morphogenesis of callus is greatly affected by the form (reduced or oxidized) and amount of nitrogen provided. Plants utilize reduced nitrogen for their metabolism; ammonia, NH_3, and amino acids (glutamine, proline, glutathione, alanine, serine, etc. and casein hydrolysates) are most commonly used in culture medium (Li, 1988). NO_3^-, a highly oxidized form of nitrogen, required in tissue culture. Plant cells cannot utilize nitrate ion directly, instead they first reduce nitrate ion into ammonium with energy expense. There are three reasons for the use of nitrate ion. The first is NO_3^- is necessary

for embryogenesis, a lack of it in medium results in abnormal embryos or a reversal of embryogenesis to callus formation (George, 2006). The second is that the single application of NO_3^-or $NH4^+$ has latent toxicity and hence a combined application of NO_3^- and $NH4^+$ can prevent the toxicity. The third is that, through adjusting pH, nitrate ion promotes the absorption of $NH4^+$ (George, 2006). Experiments have conformed that improved embryo results when both are used together rather than separately (e.g. Shetty and McKersie, 1993), and a suitable ratio of NO_3^- and $NH4^+$ is a key factor influencing somatic embryogenesis (McKersie and Brown, 1996). Like requirements for other nutrients, a suitable concentration of nitrogen is necessary for embryogenesis. A low level of nitrogen cannot meet the requirements of cell activity and growth; whereas, a high concentration of nitrogen inhibits embryogenesis due to the toxic effect of ammonium and nitrate ion (George, 2006).

2.3.3.3. Macro and microelements

Carbon and nine other chemical elements were first established as "essential inorganic nutrients" for plant normal growth (Raven *et al.,* 1992). By 1954, this family was expanded by six other members, manganese, zinc, copper, chlorine, boron, and molybdenum. These elements vary greatly in concentration, form of absorption and function in plants. However, the absence of any of these elements will result in characteristic abnormalities of growth or deficiency symptoms (Li, 1988; Raven *et al.,* 1992). They are also essential for somatic embryogenesis. For instance, potassium influences the cell K4-ATPase activity, which in turn regulates nutrient uptake and metabolite allocation (Shetty and McKersie, 1993).

Table – I

Elements	Observed forms	Usual conc. in plants cells (% of ppm/DW)	Main function
Macroelements			
Carbon	CO_2	about 44%	Component of organic compounds
Oxygen	H_2O or O_2	about 44%	Same as above
Hydrogen	H_2O	about 6%	Same as above
Nitrogen	NO_3^- or NH_4^-	1-4%	Component of amino acids, proteins, nucleotides, nucleic acids, chlorophylls, coenzymes
Potassium	K^+	0.5-6.0%	Osmosis, ionic balance, opening and closing of stomata, activator of many enzymes
Calcium	Ca^{2+}	0.2-3.5%	Component of cell walls, enzyme cofactor, involved in cellular membrane permeability; component of calmodulin, regulator of membrane and enzyme activities
Phosphorus	$H_2PO_4^-$ of HPO_4^{2+}	0.1-0.8%	Component of ATP and ADP, nucleic acids, coenzymes, phospholipids
Magnesium	Mg^{2+}	0.1-0.8%	Part of chlorophyll molecuIe and activator of many enzymes
Sulphur	SO_4^{2-}	0.05-1.0%	Component of some amino acids, proteins and of coenzyme A
Microelements			
Iron	Fe^{2+}or Fe^{3+}	25-300 pprn	Chlorophyll synthesis, component of cytochromes and nitrogenase
Chlorine	Cl^-	10-10,000 pprn	Osmosis and ionic balance and photosynthetic reactions
Copper	Cu^{2+}	4030-ppm	Activator or component of some enzymes
Manganese	Mn^{2+}	15-800 ppm	Activator of some enzymes, required for integrity of chloroplast membrane and for oxygen release in photosynthesis
Zinc	Zn^{2+}	15- 100 ppm	Activator or component of many enzymes
Molybdenum	MoO_4^{2+}	0.1-5 ppm	Nitrogen fixation and nitrate reduction
Boron	B(OH), or $B(OH)_4^-$	5-75 ppm	Influences Ca^{2+} utilization, nucleic acid synthesis, and membrane integrity

2.3.3.4. Vitamins

Like animals, plant cells require vitamins as essential intermediates or metabolic catalysts. For instance, thiamine is an essential factor in carbohydrate metabolism, nicotinic acid provides the skeleton of NAD and NADP, which are the co-factors of dehydraogenase enzymes, and pyridoxine is one of the co-factor for amino acid metabolism (Nie *et al.,* 1989). Plants produce their own vitamins; however, cultured plant tissues and cells can not produce their own requirements. As a result, it is necessary to supply vitamins in culture medium (George, 2006). The commonly applied vitamins are thiamine (Vit. B_1), nicotinic acid (niacin, Vit. PP), pyridoxine (Vit. B_6) and myo-inositol (Vit. B complex) since they play an important role in metabolism but are deficient in cultured cells and tissues (Brown *et al.,* 1995; George, 2006).

2.3.3.5. Organic additives

Organic compounds, mainly amino acids, are beneficial for somatic embryogenesis. Amino acids serve as a supplemental nitrogen source to ammonia and nitrate ion, affect metabolism, and pH of the buffer medium (George, 2006 ; Shetty and McKersie, 1993; Stuart and Strickland, 1984; Trigiano and Conger, 1987). The addition of proline and its analogs such as thioproline were reported to be the most effective among amino acids to promote somatic embryogenesis and embryo morphology (Shetty and McKersie, 1993). Serine, alanine, glutamine, arginine, lysine, asparagine and ornithine also stimulated the quality and quantity of somatic embryos (Stuart and Strickiand, 1984). Casein hydrolysates and yeast extract contains several amino acids, vitamins and other nutrients and are widely used in culture media (Brown *et al.,* 1995).

2.4. Somatic embryogenesis in *Musa*

2.4.1. Explant selection and induction

Somatic embryogenesis has been reported in more than 200 species during the last 35 yrs (Vasil, 1994). Though somatic embryogenesis in *Musa*, is subject of research since 1960s (Krikorian and Scott, 1995), the first report of somatic embryogenesis in *Musaceae* was that of Cronauer and Krikorian (1983), who obtained somatic embryos from cell suspensions derived from apices cultured *in vitro*. Somatic embryogenesis which is a basis for genetic engineering has been successful in many banana cultivars such as Bluggoe, Pelipita (ABB) (Novak *et al.,* 1989; Dhed'a *et al.,* 1991; Panis and Swennen, 1993; Escalant and Teisson, 1994), Grand Naine (AAA)

(Novak *et al.,* 1989; Escalant and Teisson, 1994; Cote *et al.,* 1996; Becker *et al.,* 2000), Mysore, Silk (AAB) (Escalant and Teisson, 1994), Rasthali (AAB) (Ganapathi *et al.,* 2001a) and Mas (AA) (Mahanom *et al.,* 2003; Wong *et al.,* 2006) using various types of explants.

The choice of explant depends on the regeneration capacity of the cells. On the basis of difference in explant types, four main methods have been developed. Novak *et al*. (1989) used the bases of leaf sheaths or rhizome fragments of plants produced *in vitro*. Dhed'a *et al*. (1991) started from thin sections of a highly proliferating bud culture placed in a liquid medium. Marroquin *et al*. (1993) established embryogenic suspension from immature zygotic embryos.

Botti and Vasil (1983) developed embryogenic cell suspension from excised zygotic embryos. Similar work was done by Vasil *et al*. (1985). Escalant and Teisson, (1988) developed somatic embryos from the seeds of banana fruit by culturing them on basal medium supplemented with plant growth regulators. Further research was carried out to find the similarity between the zygotic and somatic embryos. Escalant and Teisson, (1993) found that calli from immature zygotic embryos and somatic embryos were good for induction of embryogenic cell suspension.

Cronauer and Krikorian (1983) reported the production of somatic embryos in liquid medium from cells derived from cultured shoots of two plantain clones, Saba and Pelipita. Cronauer Mitra and Krikorian (1988) also reported regeneration of plants via somatic embryogenesis in the seeded diploid banana *Musa ornata* Robx. In their study they observed that friable yellow callus began to form near the shoot of the zygotic embryos, which further produce tiny somatic embryos. These somatic embryos were formed profusely in the presence of relatively high levels of 2,4-D.

Escalant and Teisson (1989) found that the serial sections of zygotic and somatic embryos showed perfect homology in their structures which is similar to the finding of Rodriguez *et al*. (1999). But Rodriguez described that somatic embryos do not have well developed structures, when compared to mature zygotic embryos. Domingues *et al.* (1996) selected rhizome and pseudostems of banana (*Musa spp*.) cultivars Maca, Sanicao and GN60 as an explant to initiate cell suspension in

MS medium supplemented with vitamins and dicamba. They found that regeneration capacity of these embryogenic cells were very low.

Novak *et al*. (1989) reported somatic embryogenesis in liquid medium using callus obtained from leaf bases whereas the regeneration process in *E. superbum* started with the induction of vitrification using high levels of BAP followed by callusing, and the finding was in close conformity with an earlier reports (Afza *et al.,* 1994) in the case of *E.ventricosum* (Kulkarni *et al*., 2002).

Schoofs *et al*. (1998) developed a widely applicable methodology for the initiation of embryogenic cell suspensions using scalps. Among the advantage of using scalps are that no frequent field-trips are required, unlike other methods (for instance, to collect immature flowers) and as such, virus-indexed plant material can be used and the response for embryogenesis is not season dependant. Similarly, Dhed'a *et al*. (1992) and Stross *et al*. (2005) focused on multiple meristem cultures as the starting material from which an approximate 3mm top layer (i.e., scalp) which was excised and cultured on embryogenesis induction medium. They also reported that the embryogenic induction was observed in scalp at 3-8 months and constituted of individual embryos, compact callus and/or friable embryogenic callus bearing numerous translucent proembryos (ideal embryogenic callus). Success rates in the establishment of *Musa* ECS and morphological characteristics of cell culture derived from multiple meristem culture are in accordance with results obtained in case of male flower buds (Panis *et al.,*1993; Cote *et al.,* 1996; Schoofs 1997; Strosse *et al.,* 2003).

Silvia *et al*. (2001) reported somatic embryogenesis induction in shoot meristematic apices of cv. Nanicao (AAA). Histological sections of embryogenic regions of the explant at 26 days in culture revealed the formation of meristematic regions, structures with multiple root meristems, and somatic embryos at early globular statges. Use of scalps for embryogenic response does not rely on time or season and this provides a useful tool to control the quality of the whole process (Strosse *et al.,* 2006). However, this method that uses a high dose (100 mM) of N6-benzylaminopurine (BAP) produces scalps in the EAHBs only after nine subculture cycles and is cultivar dependant. The prolonged use of a high dose of BAP has the disadvantage of producing

somaclonal variation and/or an undesirable decrease in somatic embryogenesis (INIBAP, 2000).

Wang Xiao *et al.,* (2007) reported that protoplasts of cv. Mas (AA) could divide and form cell colonies in liquid culture system contrasting previous reports that protoplasts of banana could not divide and form cell colonies when cultured in liquid medium (Panis *et al.,* 1993; Assani *et al.,* 2001). Panis *et al.* (1993) reported the plant regeneration through direct somatic embryogenesis from protoplasts of banana (*Musa* spp.). Their results proved that 50% of plant regeneration occurred from microcolonies *via* somatic embryos when high inoculation densities or nurse cell cultures were used. In the same year, Megia *et al*. (1993) regenerated plantlets from protoplast of cell suspension derived from meristematic buds in cv Bluggoe (ABB). It was also found that the protoplast developed directly into embryos without passing through callus phase. Whereas, Assani *et al*. (2001) reported the establishment and regeneration of protoplast derived cell suspension and microcalli in cv. Grand Naine.

2.4.2. Male and female buds as explant

After transition from the vegetative to the floral stage, the banana meristem initially differentiates into female hands (group of flowers inserted at the same node). At this stage, the meristem remains active and produces hands of transitional flowers and then hands of male flowers. Hence, in *Musa* immature male buds are being used as explant for producing embryogenic cell suspensions (Stover and Simmonds, 1987). Ma (1991), Escalant and Teisson, (1994), Grapin *et al*. (1996, 2000) used young male flowers as starting material for initiating suspension culture. The female flower could also be used as explant for the development of ECS but it leads to the destruction of entire bunch (Grapin *et al.,* 1998).

With *Musa* male flowers, 15 hands proximal to the terminal meristem were used as explants (Escalant and Teisson, 1994; Grapin *et al.,* 1996). For this type of explant the number of flowers per hand does not change with the row and probably size was the only deciding factor for the explant response. The response of hands of male flowers depends strongly both on genotype and the time of year when it is initiated (Escalant and Teisson, 1994).

Escalant and Teisson, (1994), Cote *et al*. (1996) and Grapin *et al*. (1998) employed male and female flowers in cultivars of the groups AA, AAA, AAB and AAAB to develop embryogenic tissues which was later used to establish cell suspension cultures. Uma *et al*. (2007) studied the embryogenic capacity of eleven commercial Indian cultivars across the genome (AB, AAA, AAB and ABB) and reported that embryogenesis through male buds is genome dependant. Such genomic dependence has also been observed in scalp cultures (Schoofs *et al.,* 1997).

Wirakarnain *et al.* (2008) developed a protocol for the establishement of competent multiple meristem cultures of banana, *Musa acuminata* cv. 'Pisang Mas' (AA) derived from male inflorescence which could be an alternative material for mass multiplication of bananas. The current trend has exploited the meristem inflorescences as explants for *in vitro* micro propagation techniques (Resmi and Nair 2007; Krikorian *et al.,* 1993) because these materials reduce the contamination rate compared to soil grown suckers.

Lemos (1994) focused on somatic embryogenesis in two genomes of banana (*Musa* spp. AAA and AAB group). del-Sol *et al*. (1995) developed embryogenic callus from immature male flowers of *Musa sapientum* and *Musa paradisiaca* using different concentrations of 2,4-D and different types of gelling agent. They also proved that phytagel was the best gelling agent and concentration of 2,4-D required for the induction of embryogenic callus varied from cultivar to cultivar. Ganapathi *et al*. (1999) used male flowers in five cultivars of banana to produce embryogenic cultures. Grapin *et al*. (2000) reported indirect embryogenesis through the development of induced embryogenically determined cells as explained by Ammirato *et al.* (1987). Daniel *et al*. (2002) developed cell suspension from calli with embryogenic structures obtained from young male flowers of banana. Antra gosh *et al*. (2009) developed embryogenic cell suspension (ECS) using immature male flowers of cultivar Robusta. The embryogenic tissue can be obtained from female flowers or male flowers but both of them resemble in characters (Escalant and Teisson, 1994; Grapin *et al.,* 1996).

Marroquin *et al.* (1993) mastered the regeneration of embryogenic suspension by using zygotic embryos and Ma (1991) reported regeneration using embryogenic suspension initiated from young male flowers. More efforts have been taken to

standardize the regeneration protocol for Cavendish banana. Navarro *et al*. (1997) reported plant regeneration via somatic embryogenesis in Grand Naine which include the combination of growth regulators like 2,4-D, IAA and NAA. Improved system for the mass propagation via somatic embryogenesis was described in cv. Grand Naine (Chong-perez *et al.,* 2005) and hybrid FHIA-18 (AAAB) (Gomez kosky *et al.,* 2002). And further secondary multiplication of somatic embryos was achieved in liquid medium in rotary shaker from which high regeneration of plants (89.3%) was obtained in one month. Khalil *et al*. (2002) studied and standardized the regeneration protocol for secondary somatic embryogenesis in cv. Dwarf Brazilian. Ganapathi *et al*. (2001b) proved that somatic embryos can be used as explant for the production of synthetic seeds. It was also reported that germination frequency of encapsulated embryos varied considerably on different gel matrices and subtrates used for plant development.

2.4.3. Factors controlling somatic embryogenesis

According to Litz and Jarrett (1991), plant tissues have the ability to form callus under *in vitro* condition but relatively few explants have the ability to produce callus with embryogenic structures. By subjecting the explant culture to unusual conditions (stress), might be feasible to eliminate or lessen the proper gene expression for the development of somatic embryogenesis. Based on this, several methods have been used to induce somatic embryogenesis, such as heat, increasing the hypochlorite ion concentration, anaerobiosis, low temperature (4°C), high osmotic pressures and exposure to auxin (Merkle *et al*., 1995). Besides time, the efficiency of somatic embryogenesis and plant regeneration system through cell suspension cultures is determined by the following key factors: (i) the availability of embryogenic competent explants, (ii) the nature and frequency of embryogenic response, (iii) the success rate of suspension initiation, (iv) the plant regeneration frequency and (v) trueness-to-type (Schoofs *et al.,* 1999).

Embryogenic response and high regeneration ability were shown to be under genetic control, sexually transferable to less responsive genotypes through hybridization in maize and wheat (Tomes and Smith, 1985; Higgins and Mathias, 1987), alfalfa, soybean, cotton and potato (Seitz and Bingham, 1988; Sonnino *et al.,* 1989). Brown and Atanassov (1986) and Parrot *et al.* (1989) found that cultivars showing high levels of embryogenesis descended from a few specific ancestral

germplasm sources. Embryogenic responses can also vary among cultivars or even between individuals of a given cultivar (Chen *et al.,* 1987; Szabados *et al.,* 1987; Komatsuda and Ohyama, 1989; Feirer and Simon, 1991).

Medium composition or other culture effects, was found to affect embryogenic responses of less embryogenic genotypes (Chen *et al.,* 1987- Brown, 1988). Monitoring these potential of culture would be useful for the stable production of somatic embryos (Ibaraki *et al.,* 1998). Vasil (1987) and Close and Gallagher-Ludeman (1989) commented that in case monocots, highly recalcitrant plants or genotypes can be induced to undergo morphogenesis in culture, when suitable explants at defined developmental stages are excised from plants under optimal condition, and when the appropriate growth regulators and media are used.

Induction of callus towards embryogenesis is largely dependent on a reduced auxin-cytokinin balance in the culture media (Michaux Ferriere and Schwendiman 1992). But still auxin is considered to be the most important hormone in regulating somatic embryogenesis (Cooke *et al.,* 1993). Both the endogenous contents and the application of exogenous auxins are determining factors during the induction and expression phases of somatic embryogenesis. The choice of auxin is very important in the induction process and can affect the frequency and morphology of somatic embryos (Levi and Sink 1991, Rodriguez and Wetzstein 1994). Among 65 dicot species reviewed by Raemakers *et al.,* (1995), somatic embryogenesis was induced in 17 species on hormone-free media, in 29 species on auxin-containing media and in 25 species on cytokinin-supplemented media. Among auxins, the most frequently used was 2,4-D (49%) followed by naphthalene acetic acid (27%), indole- 3-acetic acid (IAA) (6%), indole-3-butyric acid (6%), Picloram (5%) and Dicamba (5%). In the case of cytokinins, N6-benzylaminopurine was used most often (57%), followed by kinetin (37%), zeatin (Z) (3%) and thidiazuron (3%).

The low production of callus with embryogenic structures remains as one of the major constraints in the process of somatic embryogenesis which is directly influenced by the concentrations of 2,4-D used (Dion daniels *et al.,* 2002). It has been reported that the culturing of explants in 2,4-D-containing medium increases the endogenous auxin levels in the explants (Michalczuk *et al.,* 1992). It has also been observed that polar

transport of auxin is essential for the establishment of bilateral symmetry during embryogenesis in dicotyledonous somatic (Schiavone & Cooke, 1987) and zygotic (Liu *et al.,* 1993) embryos, and more recently it was also demonstrated for monocotyledonous zygotic embryos (Fischer & Neuhaus, 1996). Cote *et al.* (1996) and Gomez *et al.* (2000) used a concentration of 4.5 μM 2,4-D in the multiplication of cell suspensions of Grand Naine (AAA) and FHIA-18 (AAAB), respectively, while the highest cell growth was obtained at 13.5 μM 2,4-D.

Ivanova *et al.,* (1994) found a relation between endogenous levels of growth regulators especially IAA levels, and the ability to respond to 2,4-D induction treatment. Fast embryogenic lines have endogenous IAA levels, 4 times higher than that of slow embryogenic lines and 30 times higher than that in non embryogenic lines. Non embryogenic lines moreover were found to have high endogenous levels of abscisic acid (ABA). Once the stimulus for the further development of the somatic embryos is given (i.e., through reduction or removal of 2,4-D from the culture medium), those levels must be reduced, to allow the establishment of the polar auxin gradient. If the levels are extremely low or high, or if they do not diminish after the induction treatment, the gradient cannot be formed and thus somatic embryogenesis cannot be expressed.

One of the most powerful aspects of somatic embryogenesis which allows its application in mass propagation and gene transfer (Merkle *et al.,* 1995). These processes have been propagated several terms, as secondary embryogenesis, recurrent or repetitive (Gomez, 1998). Repetitive embryogenesis can occur in the absence of an exogenous auxin. This process is called autoembryogenesis and is sometimes referred to as proliferation or mass propagation (Merkle *et al.,* 1995).

Cytokinin also plays a major role in embryo formation of banana ECS (Novak *et al.,* 1989; Dheda *et al.,* 1991; Ma 1991; Cote *et al.,* 1996; Grapin *et al.,* 1996). Arinaitwe (1999) also reported an increase in bud proliferation rate as the concentration of TDZ was increased from 0.045 to 6.81 mM. The higher cytokinin activity of TDZ was attributed to its ability to accumulate in cultured tissues to act as endogenous cytokinins (Huetteman and Preece, 1993). Nahamya (2000) reported higher multiple bud proliferation and earlier scalp formation in EAHBs with TDZ than with BAP when

the two cytokinins were used at concentrations of 10 mM and 100 mM, respectively. Similar work was carried out by Sales *et al*. (2001), in which effects of various benzyladenine concentration on scalp initiation was studied to produce embryogenic callus from them.

The choice of the explant might be the key factor that determines failure or success of an embryogenic protocol (Brown *et al.,* 1995; Krishnaraj and Vasil, 1995). In monocots, cells differentiate rapidly, thereby loosing totipotency. Only meristematic cells and partially differentiated cells, that are developmentally uncommitted, reveal mitotic and regeneration ability (Krishnarraj and Vasil, 1995). The presence of mature and more differentiated tissues appears to inhibit the expression of embryogenic competence in uncommitted cells (Vasil, 1987). Merkle *et al.,* (1995) relate the explant type and its developmental stage or age again to the epigenetic 'distance' of tissue cells from the embryogenic state. For pre-embryogenic determined cells and tissues (PEDCs), the use of a cytokinin only may be sufficient to induce embryogenic determined cells (IEDCs), an auxin, or auxin and cytokinin are needed. Cells in tissues can range anywhere between PEDC and IEDC, and often the cell population is very heterogenous.

Number of subcultures is another important factor controlling the rate of proliferation and friability of the callus produced. Fitchet (1987) reported that friability of callus increased with number of subcultures of callus. Formation of ideal calli also depends on the age of the explant and position of the explant. In banana cultivars, from 5^{th} to 9^{th} whorls formed more callus with high frequency of somatic embryogenesis than the 10^{th} to 14^{th} whorls (Gomez Kosky *et al*., 2001).

Mavituna and Buyukalaca (1996) described those oxygen uptake rates of cultures was the highest being in embryogenic suspension cultures and the lowest during embryo maturation. George *et al.* (2006) reported that the suspension induced in liquid medium will have varying regeneration capacities. Their results further indicated that quality of an embryogenic cell suspension decreased with the increased number of subcultures. This results in an increased probability of contamination and decreased growth rate and regeneration capacity, due to the fast growing dense cells rich in starch. This is similar to the findings of Ma *et al*. (1989) who also proved that the plant

regeneration frequency was dependent upon the cultivar and was also influenced by growth factors present in the culture medium. The suspension cell cultures retained their regeneration ability for more than a year.

Even though callus can be produced in the absence of light both from the vegetative and reproductive tissues in MS medium with 2,4-D, regeneration and germination capacity of the embryogenic cells depended largely on genotype and nature of explant. Quantitative regeneration is based on weight measurements and quantity of germinating embryos and plants per milliliter of settled cell volume (SCV). Hence, the germination capacity of embryogenic cells using different explants is same. Sharma and Millam (2004) stated that the key factor to the success of any somatic embryogenesis system is the ability to discriminate SE-specific cellular structures from those emerging through an organogenic route whereas, the response of hands of male flowers depends strongly on both the genotype and the time of the year when it was cultivated (Escalant and Teisson, 1994).

2.4.4. Embryogenic cells and their embryogenic capacity

Examination with light and electron microscopy (Halperin and Jensen, 1967; Street and Withers, 1974) resulted in the definition of embryogenic cells: rounded cells with dense cytoplasm rich in starch reserves, a large nucleus with a single enlarged nucleolus. But Grapin *et al*. (1996) for the first time reported that cells devoid of starch and with only protein reserves also has embryogenic potential. William and Maheswaran (1986) mentioned that suspension with a great number of actively dividing spherical cells and heterogenous, irregular cell masses can be an indicator of the embryogenic condition of cell suspensions. On the other hand histological study revealed that the yellow globular compact structures developed from non-embryogenic suspensions were vacuolated with no accumulation of starch or protein reserves which was believed to be the factor of poor redevelopment of somatic embryos (Mahanom jalil *et al.,* 2008).

Image analysis techniques are promising method for evaluation of cultures because it can non-destructively monitor the cultures and provide objective indices for visual information. Microscopic image analysis for suspension culture could be used to select proembryogenic masses. In some cases, the area was used directly as a cell

quantitative parameter; otherwise the area was correlated to normal quantitative parameters, such as fresh weight, dry weight, packed cell volume (PCV) and the number of cells (Ibaraki and Kurata, 2001).

Numerous internal cell divisions led to the formation of proembryos, distinct from each other, at the beginning of the embryos development phase. This probably indicates the unicellular origin of the embryos. It was also observed that embryogenesis could occur from nodules, as shown by Dhed'a *et al*. (1991) in respect of cv Bluggoe.

Culture density of cell suspensions could be another important factor that affects somatic embryogenesis. A high cell number is required to induce their transition into embryogenic cell clumps (Toonen *et al.,* 1994). Cell density seems to have an impact on cell proliferation and maintenance of embryogenic characteristics. Best result were obtained by diluting the suspension to 1/3. Toonen *et al*. (1994) reported on cell densities as high as 10,000 cells/ml, needed to induce cell divisions in state 0 cells (partially embryogenic state). Similarly, Nomura and Komamine, (1986) proved that high cell density (105 cells/ml) is required for the formation of embryogenic cell clusters from single cells (Nomura & Komamine, 1986), whereas lower cell density (2 x 104 cell/ml) favors the development of embryos from embryogenic cells (Fujimura & Komamine, 1979). Gomez *et al.* (2000) stated that by using low densities in the hybrid cultivar FHIA-18 (*Musa* sp. AAAB) there is greater accumulation of reserve substance and lower multiplication.

Novak *et al.* (1989) found that both subculture interval and incubation temperature, influence the relative frequency of embryogenic cells, compared to non-embryogenic and partially embryogenic cells. When ECSs are not regularly subcultured, the suspension becomes mucilaginous (Nayak and Sen, 1989; Vasil and Vasil, 1982). The same observations were made in *Musa* ECSs (Panis, 1995). Upon culture time, cultures loose their embryogenic characteristic. The colour of the suspension changes, and cell are less densely packed. Histological studies revealed starch accumulation and vacuolation in older cells. To avoid this, media should be refreshed before reaching the end of the exponential phase of the growth curve. Ho and Vasil (1983) also reported on methods of subculturing, affecting the growth rate of cells and the density of cell clumps in sugarcane suspension cultures.

Daniels *et al.,* (2002) stated that the embryogenic clusters release the cells in liquid medium upto 15th day of culture, after which there was no increase in cell growth and thereafter a decrease in the total number of cells in the suspension. This was due to cell death caused by high cell density and the oxidation of phenols. Further studies proved that structure of the cell suspension and the rate of cell growth were found to be dependent on the cultivar regardless of the hormone treatment in the induction medium. Cultivars '*Musa*kala', 'Kibuzi' and 'Mbwazime' produced cell culture of clustered and aggregated cells and high cell numbers, which are a prerequisite for embryo cells development (Sadik *et al.,* 2007).

Domergue *et al*. (2000) reported five different types of cellular aggregates in embryogenic cell suspension of cv. Grande Naine. Type -1 corresponded to isolated cells or small aggregates. Type-2 was composed of embryogenic cells. Type-3 can be distinguished from type -2 due to the presence of peripheral proliferation zones with embryonic cells. Type-4 was composed of protodermic masses histologically comparable to proembryos. Type-5 composed of central zone meristematic cells and of an external zone of starchy cells.

In order to improve the synchrony of embryo development, a fixed cell aggregate size is often used to select proembryogenic masses (PEM) as materials for embryo production. However, a large variation in the size of PEMs used for somatic embryogenesis has been reported. Although it depends largely on plant species, a wide variation is sometimes observed in the same species. Large PEMs were reported to produce multiple embryos (Halperin and Jenson 1967; Osuga and Komamine, 1994), and this may cause asynchrony in the culture (Ibaraki *et al.,* 2000).

For obtaining high quality and homogeneous embryos, sorting embryos on the basis of embryo quality, i.e., conversion potential is an effective technique. To obtain high conversion rates, it is often been necessary to select embryos manually (Molle *et al.,* 1993; Paques *et al.,* 1995; Gupta *et al.,* 1999). While plating embryogenic cells in regeneration medium, the number of embryos obtained per volume of plated cells is the key criteria by which the quality of a suspension could be estimated (Grapin *et al.,* 1996; Cote *et al.,* 1996; Strosse, 2005). Reinert and Yeoman (1982) described method to determine the growth of cell lines using packed cell volume (PCV). They reported

that during each subculture of cell suspension, the initial density was adjusted to 3% of the PCV in 15 ml of culture medium.

Novak *et al*. (1989) reported numbers of 20-30X10^{3} ^{s} somatic embryos in suspensions of diploid and triploid cultivars, Cote *et al*. (1996) obtained 370X10^{3} somatic embryos per ml of plated packed cell volume of 10% in Grand Naine (AAA). The somatic embryo diameter in the cell suspensions varied from 0.5 to 1.2 mm with an average size of 0.86± 0.25 mm. The weight of these somatic embryos ranged from 0.65 to 0.90 mg, depending on the development stage (Gomez Kosky *et al.,* 2002).

An indication for somatic embryos formation was the presence of protoderm, the outermost layer of a developing embryo. This fact is supported by West and Harada (1993) who stated that during embryogenesis, the first tissue that can be identified histologically is the protoderm. Similar results was obtained by Mahanom jalil *et al.* (2008) and reported that histological sections of somatic embryos in *M. acuminata* cv. Mas (AA) showed clear presence of protoderm surrounding the densely meristematic embryogenic cells with the accumulation of starch reserves. Recent study by Sharma and Millam (2004) confirmed the presence of protoderm since the early-globular stage in somatic embryogenesis.

2.4.5. Field performance of tissue culture banana plants

The advantages of *in vitro* micropropagated banana plants included higher rates of multiplication, production of disease free planting material and small space required to multiply large numbers of plants.

Field performance of these micropropagated plants has been reported by a number of authors. Nokoe and Ortiz (1998) suggested the optimum plot size for banana field trials. It was observed that 16 ± 3 plants per plot were needed to evaluate the growth characteristics and yield potential of the cultivars. The recommended optimum plot size consisted on average, 13 ± 3 plants per plot for the plant crop, and 15 ± 2 plants per plot for the ratoon crop. Smith and Drew (1990) reported that tissue culture plants had bigger pseudostem and retained more healthy leaves than those originating from suckers.

Robinson *et al.* (1993) achieved 20.4% higher yield than conventional plants, due to larger bunches and a shorter cycle to harvest. On the other hand *in vitro* derived plants of plantain (*Musa* spp. AAB) did not show any higher yield (Vuylsteke and Ortiz 1996) and more phenotypic variation was observed in tissue culture plants.

Field performance of *in vitro* raised variants was compared with true-to-type plantains to evaluate their horticultural traits (Vuylsteke *et al.,* 1996). Significant variation was observed for plant and fruit maturity, leaf size, yield and its components but not for leaf number, plant height, or suckering. Three of the four somaclonal variants were horticulturally inferior to the original clone from which they were derived. Only one variant which resembled an existing cultivar, out yielded the true-to-type clone. However, its fruit weight and size were lower. Optimum plantlet size for tissue culture banana plants was studied by Cote *et al.* (2000). They reported that small plants of 100 mm took three weeks longer to harvest and had six percent lower yields as compared to 300 mm size. Plants of 500 mm size showed slightly lower yields as compared with 300 mm plants. It was advisable that 200 mm plants should be planted at least 100 mm below soil surface, preferably in a furrow.

Cote *et al.,* (2000) compared banana plants originated from embryogenic cell suspensions and plants produced by the conventional *in vitro* budding multiplication method. There were no significant differences between the plants produced by either micropropagation techniques for the plant height and circumference, the length of the reference leaf, the number of nodal clusters of the inflorescence and fruits, the bunch weight, the period of time between planting and flowering, and between planting and harvesting. This study showed that banana plants with agronomical behavior similar to those produced by the conventional *in vitro* budding method could be regenerated from embryogenic cell suspension.

2.4.6. Somaclonal variation

Tissue culture techniques were considered to generate exact copies of the parent plant (Denton *et al.,* 1977; Wright, 1983). Somaclonal variation, however, has been identified in a vast number of plant species and occurs in both sexually and asexually propagated plants and at all ploidy levels (Al-Zahim, 1996). Somaclonal variation is one of the great stumbling blocks for present day micropropagation and plant

regeneration *via* somatic embryos (De Klerk, 1990). It also hampers the application of biotechnological breeding techniques such as somatic hybridisation for recombining genomes of sexually incompatible species, modification of plants by recombinant DNA techniques and anther cultures to speed the attainment of homozygosity (Al-Zahim, 1996). Though reports exist on the selection of useful genetic variants induced by tissue culture (Larkin and Scowcroft, 1983; Sun *et al.,* 1983; Jordan and Later, 1985; Smith and Drew, 1990), most somaclones reveal decreased vigour, yield etc. (Johnson *et al.,* 1984; Lee and Philips, 1987; Jackson and Dale, 1989).

The term 'somaclonal variation' was introduced by Larkin and Scowcroft (1981) to describe the genetic variation in plants regenerated from any form of cell culture. It is believed that somaclonal variation occurs only in plants regenerated from adventitious or newly formed apical meristems (Hussey, 1983) whereas epigenetic (i.e. non-genetic) changes can also be observed in plants originating from pre-existing apical/axillary meristems (De Klerk, 1990). In contrast to somaclonal variation, epigenetic variation as a physiological response to stress conditions, is not heritable and is reversible during the life of a plant. Severe environment stress that lies beyond the capacity of plants to adopt by epigenetic changes, can cause 'genome shock' that activates transposons leading to meitiotically and mitotically transferable genetic changes (McClintock, 1984). Somaclonal variation was found to be induced upon stress application in flax (Cullis and Cleary, 1986). On the contrary, Heindorff *et al.,* (1987) found that stress pretreatment of *Vicia faba* roots, rendered these meristem less susceptible to mutagenesis.

Somaclonal variants are an expression of pre-existing variation in the source plant or due to *de novo* variation via undetermined genetic mechanisms, likely to occur in tissue culture (Larkin and Scowcroft 1981). Effects of biological (genotype, explant type), medium (plant growth regulators) and physical (duration of cultue) factors on somaclonal variation have been noted, yet the basic knowledge remains fragmentary (De Klerk, 1990). Somaclonal variation is more likely to arise from polysomatic species and tissues, from older tissues, from highly differentiated structures such as nutritive and storage organs, and would be higher in species with large genome size, which increases the chance of replication errors (Bhatia *et al.,* 1985).

The role of certain plant growth regulators like 2,4-D in somaclonal variation is highly debated. The most widespread view is that 2,4-D would cause abnormalities by stimulating rapid disorganized growth, without being directly mutagenic (Bayliss, 1980). Bayliss (1980) found that NAA and 2,4-D cause chromosomal aberrations only at very high concentrations (50 mg/l), and not at concentrations that are being used routinely in tissue culture (<10 mg/l). It was also reported that 2,4-D induced changes in the cell cycle, and increased the frequency of sister chromatid exchange at relatively low concentrations (Dolezel and Novak, 1986, Murate, 1989). Auxins are furthermore believed to affect the ploidy level in callus cells (Ghosh and Gadgil, 1979), and increase the frequency of aneuploidy and polyploidy (Sree Ramulu *et al.,* 1983).

In general, the chance of variability increases with culture time or the number of successive multiplication (McCoy *et al.,* 1982; Cote *et al.,* 1993). Aberrations were most often linked with a progressive increase in the ploidy and aneuploidy of cells, resulting in the gradual loss of morphogenic potentialities (Murashige and Nakano, 1967). It has been demonstrated that mutations and changes in ploidy, can take place early in culture, and as such are not necessarily caused by long culture time (Torrey, 1959; Benzoin and Phillips, 1988). Polyploidisation is caused by spindle failure, chromosome lagging at anaphase or spindle fusion in binucleated cells (Mitra and Steward, 1961). Aneuploidy seems to be caused by non-disjunction, lagging chromosomes, non-polar anaphase, and aberrant spindle (D'Amato, 1978), and is believed to be tolerated better in polyploid than in diploid species (Al-Zahim, 1996). Other changes in DNA structure involve chromosome breakage, chromosomal translocation, partial duplication and deletion, single base changes, and activation of silent transposable elements (De Klerk, 1990, Al-Zahim, 1996).

2.4.6.1. Somaclonal variation in banana

Somaclonal variation is a common phenomenon in both *in vitro* and *in vivo* propagated *Musa* plants. Such somolcones (off-types) are more common in bananas and plantains regenerated *in vitro* than in conventionally propagated *Musa* (Drew and Smith, 1990; Smith, 1988; Vuylsteke *et al.,* 1988; Vuylsteke and Oritz, 1996). In banana and plantain, the off-types most often observed are concerned with variations in stature, foliage, colour, and morphology of pseudostem and reproductive organs (Israeli *et al.,* 1995). Dwarfism is the most common variant in the Cavendish sub-group and

was found to account for 75-90% of the total variants (Stover, 1987). The occurence of two different variations on the same plant is not rare (Israeli *et al.,* 1991; Vuylsteke *et al.,* 1991). In micropropagated 'False Horn' plantains, variation in inflorescence type in the form of reversion to a typical 'French' plantain bunch type accounts for 40 – 100% of the total variability (Vuylsteke *et al.,* 1988).

Hwang and Ko (1987) identified somoclonal variants of the cultivar 'Giant Cavendish' with putative field resistance to Fusarium wilt, but all had inferior horticultural characteristic, including poor yield and low fruit quality. Variants were mostly inferior to the 'Cavendish' clone from which they were derived, in that bunch and fingers of the somaclones were smaller and of little commercial value (Hwang and Ko, 1987; Smith and Drew, 1990; Stover, 1987). However, further evaluation among *in vitro* or sucker-propagated clones of these resistant variants resulted in the identification of plants with improved traits when compared to the original variant, yet slightly low yielding than the true-to-type cultivar (Hwang and Ko, 1987).

Vuylsteke *et al.,* (1996) gave a comprehensive report on the agronomic performance of some somaclones obtained from the 'False Horn' plantain cultivar. The variants differed from true-to-type in inflorescence morphology. Variation was also observed in quantitative characters like plant and fruit maturity, leaf size, yield and yield components. Only the 'French reversion' variant, which resembled an existing cultivar, out yielded the true-to-type. However, its fruit weight and size, which affect consumer preference were lower. A better understanding of the nature and extent of somoclonal variation is necessary to assess its importance in *Musa* improvement. Hence, it would be useful to devise method to identify useful somalconal variations among large populations of variants (Evans and Sharp, 1986; Karp and Bright, 1985; Larkin and Scowcroft, 1981).

Dhed'a (1992) and Grapin (1995) were the first authors to report on somaclonal variations in banana plants regenerated from embryogenic cell suspensions. Dhed'a *et al.,* (1991) reported on 5-10% abnormal somatic embryos recovered from a 'Bluggoe's suspension, which inspite of their abnormality could grow into normal plants. Out of 140 plants tested in the field, only one off-type (0.7%) with retarded growth and distorted leaves was found. Grapin (1995) reported 16-22% somaclonal

variants among plants regenerated from a 'French Sombre' suspension, whereas all plants obtained *via in vitro* clonal propagation were normal. Abnormalities encountered, were mosaic variants (partial discoloration of the leaf) with dropping leaves often revealing retarded growth. As variants with variegated leaves or with deformed laminae tend to revert to the source plant-type in the second or even the first cycle (Israeli *et al.,* 1991 and 1995; Vuylsteke *et al.,* 1991), banana plants need to be observed for at least three cycles, before a definite statements on off-type frequency can be made. Stover *et al.* (1987) stated that less than 5% off-types, are commercially acceptable.

Smith (1988) reviewed the factors influencing somaclonal variation in bananas and divided them into intrinsic and culture-induced factors. Genetic changes induced during micro-propagation can be influenced by choice of explant, the composition of culture media (nature and concentration of phytohormones), number of subcultures or length of time in culture, and the level of dedifferentiation, the tissues undergo in the culture. Intrinsic factors include the genetic stability of the cultivar or genotype used in micropropagation. A direct relationship exists between time spent in culture and the rate of somaclonal variation. To reduce the problems related to sub culturing, a cryopreservation protocol was developed, making it possible to store embryogenic cells for unlimited periods (Panis and Thinh, 2001). Schukin *et al.* (1997) exploited the embryogenic capacity of Grand Naine and their effects on somaclonal variation. They described that the rates of somaclonal variation were 0.5% and 3.6% respectively.

2.4.6.2. Use of molecular markers to detect variation

Tissue culture induced variations can be determined at the morphological, cytological, biochemical, and molecular levels with several techniques. Molecular markers suitable for generating DNA profiles have proved to be an effective tool in assessing the genetic stability of regenerated plants. These markers are not influenced by environmental factors and generate reliable, reproducible results. DNA- based markers most frequently in use include restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD). Among these techniques, RAPD is becoming a widely employed method in the detection of genetic diversity since it has the advantage of

being technically simple, quick to perform and requires only small amounts of DNA (Williams *et al.,* 1990).

The genetic integrity of micropropagated plants can be determined with the use of various techniques. RAPD technique is employed, as it has the advantage of simplicity, rapidly and is of relatively low cost. It requires very little plant material and quick DNA extraction protocols are suitable (Rafalski *et al.,* 1999). Various authors have found that RAPD technique useful in examining tissue culture induced variation. *In vitro* culture can induce genetic variability, termed somoclonal variation (Larkin *et al.,* 1981). While this is considered as a problem within the use of RAPD markers, somaclonal variants were identified in *Prunus persica* (Hashmi *et al.,* 1997) and *Phalaenopsis* (Chen *et al.,* 1998). The clonal fedility of micropropagated plants has also been determined with RAPD technique in *Pinus thunbergii* (Goto *et al.,* 1998), Lilium (Varshney *et al.,* 2001) and *Tylophora indica* (Jayanthi and Mandal, 2001).

Phenotypic changes associated with genetic alterations were reported among tissue culture derived plants of several species from *Pelargonium, Ananas, Phalaenopsis, Arachis* and *Musa* (Cassels *et al.,* 1997). Identifying variants during the early stages of plant development is essential to avoid propagation of mutant plant in species with extensive growing periods, like forest and fruit trees (Jaligot *et al.,* 2000). Random amplified polymorphic DNA (RAPD) is an effective molecular technique for screening for genomic alterations among tissue culture derived plants (Heinze and Schmidt, 1995). In case of dicotyledonous woody plants, there are few reports of RAPD analysis being applied to monitor genomic changes (Heinze and Schmidt, 1995). Instead this method has been applied extensively to evaluate natural genetic diversity among plants (Deng *et al.,* 1995). Chromosome evaluation is another approach that has been used successfully to study genetic abnormalities among tissue culture derived plants (Straus, 1954; Maluszynska *et al.,*1998). Most studies indicate that the common chromosomal abnormalities found are polyploidy, aneuploidy and breakages at heterochromatic regions (Fourre *et al.,* 1997).

Genetic fidelity of micropropagated *Arachis* plants has been assessed by Random Amplified Polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphism (AFLP) analysis, and no polymorphism was detected at the genomic

regions tested (Gagliardi *et al.,* 2003). RAPD analysis using arbitrary oligonucleotide primers allows detection of changes in the plant genome at the DNA level. This technique has been used to analyze plants regenerated from cultured cells and tissues for the detection of somaclonal variants (Vidal and de Garcia, 2000). According to Tingey and del Tufo (1993), polymorphism is due to the presence or absence of an amplification product from a single locus. Apart from the marker DNA band, the differences in band intensity between variant and normal clone has also been detected.

Randomly amplified polymorphic DNA (RAPD) (Willams *et al.,* 1990) and inter simple sequence repeats (ISSR) (Zietjiewicz *et al.,* 1994) markers have proven to be efficient in detecting genetic variations. Both RAPD and ISSR markers have been successfully applied to detect the genetic similiarities or dissimiliarities in micropropagated material in various plants (Martin *et al.,* 2006).

While experimenting with plantains (AAB), Cronauer and Krikorian (1983) observed that the frequency of the occurrence of variants in any variety was confined to individual primary explants rather than the genotype. Another common undesirable somoclonal variant observed in 'Cavendish' banana was the mosaic type, where the heterogeneity and incidence of variation were epigenetic (Israeli *et al.,* 1991). All these reports inferred that such somoclonal variations were the resultants of three way interactions of initial explants, the culture conditions, and the genotype. The polymorphism in amplification products could result from changes in either the sequences of the primer binding sites or from changes that could have altered the sizes of the DNA fragments.

Inter simple sequence repeat (ISSR) is a PCR based method, which involves amplification of DNA segments present at an amplifiable distance between two identical microsatellite repeat regions oriented in opposite direction. The technique uses microsatellites, usually 16–25 base pair long primers in a single primer PCR reaction targeting multiple genomic loci to amplify mainly the inter-SSR sequences of different sizes.

The primers used can be either unanchored (Gupta *et al.,* 1999; Wu *et al.,* 1994) or anchored at the 3' or 5' end with 1 to 4 degenerate bases extended into the flanking

sequences (Zietkiewicz *et al.,* 1994). The microsatellite repeats used as primers can be di-nucleotide, tri-nucleotide, tetra-nucleotide or penta-nucleotide. ISSRs have high reproducibility possibly due to the use of longer primers as compared to RAPD, which permits the subsequent use of a high annealing temperature (45–60° C) leading to higher stringency.

The sources of variation in ISSR markers could be: (1) mutations at the priming site (SSR), which could prevent amplification of a fragment as in RAPD markers and thus give a presence/absence polymorphism; (2) an insertion/deletion event within the SSR region or the amplified region would result in the absence of a product or length polymorphism depending on the amplifiability of the resulting fragment size. The ISSR marker technique is simple, quick, and efficient. The primers are long resulting in high stringency and hence reproducibility. The amplified products are usually 200-2000 bp long and amenable to detection by both agarose and polyacrylamide gel electrophoresis. The technique is not without limitations. For instance, there is a possibility as in RAPD that fragments with the same mobility may originate from non-homologous regions, which can contribute to some distortion in the estimates of genetic similarities (Sanchez *et al.,* 1996).

Vendrame *et al.* (1999) reported that genetic variation in a culture line could be affected more by the genotype than by the period in culture. This genetic instability may be a risk associated with the application of in vitro culture techniques for germplasm handling and storage. Conversely, somaclonal variation may provide another source of novel and useful variability (Vuylsteke, 1998), which can be used in genetic improvement of banana (Sahijram *et al.,* 2003). Rout *et al.* (2009) assessed the quality of *in vitro* derived regenerates with ten ISSR primers. The banding pattern of PCR amplified product from micropropagated plantlets was found to be monomorphic for most of the primer tested. Most of the primers showed identical DNA profiles as compared with mother plant. Very few plants showed variation at the DNA level but morphologically they were identical with each other. Shenoy and Vasil (1992) reported that micropropagation through meristem culture are generally less subjected to genetic changes that might occur during cell differentiation under *in vitro* conditions.

Ray *et al.* (2006) highlighted the genetic stability of the micropropagated plants of three banana cultivars i.e. Robusta (AAA), Giant Govenor (AAA) and Martaman (AAB) by using 21 RAPD and 12 ISSR primers. They found three somaclonal variants from 'Robusta' and three from 'Giant Govenor". Harirah and Khalid (2006) used eighteen arbitrary decamer primers to study the clonal fedility of *Musa acuminata* Cv. Berangan. They found that all the regenerated plants were monomorphic. No somaclonal variation was detected. In some cases, regeneration process is prone to somatic variation resulting in off-types as in case of *Populus termuloides* (Rahman and Rajora 2001) and tea clones (Devarumath *et al.,* 2002). Variation is induced by different genetic and epigenetic mechanisms that are likely to be reflected in the banding pattern developed by employing different marker system. However, the reliability and efficiency of molecular markers in detecting large scale genome arrangements have been frequently questioned. Since simple sequence repeat based primer target the fast evolving and hyper variable DNA sequence, ISSR markers are considered suitable to detect variation among tissue cultured produced plants (Rahman and Rajora, 2001; Ray *et al.,* 2006; Joshi and Dhawan, 2007). The thorough scanning of the available literature indicates that markers could be well exploited in the study the genetic fidelity of ECS derived plants.