CHAPTER 2

REVIEW OF LITERATURE
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2.1 Tissue Culture

Plant tissue culture is the technique in which isolated plant cells, tissue, organs or even entire plants are grown on nutrient medium, under aseptic condition in test tubes/flasks. Production of quality plants in large number, establishment of new hybrids, and multiplication and preservation of germplasm are some benefits which have been derived from application of these techniques (Sagawa 1991).

The technique of tissue culture is historically linked to the discovery of Schwann’s (1839) Cell theory, which proposed that each living cell of an organism, if provided with proper environment would be capable of independent development and gave birth to the concept of ‘totipotency’. A totipotent cell is one that is capable of developing by regeneration into a whole organism and this term was probably coined by Morgan in 1901 (Krikorian and Berquam, 1969). The famous German botanist Haberlandt (1902) first attempted to cultivate isolated plant cells in vitro on an artificial medium although he did not succeed due to his wrong choice of explants. The first true plant tissue cultures were obtained by Gautheret (1934,1935) from cambial tissue of Acer pseudoplatanus.

The first plant tissue cultures, in the sense of long-term cultures of callus, involved explants of cambial tissues isolated from carrot (Gautheret
1939; Nobecourt 1939) and tobacco tumor tissue from the hybrid Nicotiana glauca x N.langsdorffii (White 1939)

The successful establishment and growth of plant cells in vitro generally is determined by the choice of the right plant material and the formulation of satisfactory nutrient medium (White 1951).

2.2 Micropropagation techniques for banana

2.2.1 Shoot-tip/meristem culture

Shoot-tip culture has already been applied successfully to the rapid clonal propagation of many different Musa species and cultivars and as a means of germplasm exchange and conservation (IBPGR 1989). Although other in vitro culture system also hold great promise, shoot-tip/meristem culture is the only method yet known by which plants have been regenerated in a consistent manner. Moreover, it is the technique par excellence to minimize somaclonal variation, which is an important consideration when handling crop germplasm in vitro (Scowcroft 1984). Other important applications are the eradication of diseases and the conservation and exchange of germplasm (De Langhe 1984).

The earliest reports of banana plants produced by in vitro shoot-tip culture came from Taiwan, China, in the early 1970s (Ma and Shii 1972, 1974 and Ma et al. 1978). Berg and Bustamante (1974) used meristem culture combined with heat therapy, to produce virus free bananas in Honduras. A team at the University of the Philippines produced banana shoots in vitro for mutation induction by irradiation (De Guzman et al. 1975, 1980).
Vassey and Rivera (1981) used meristem culture technique for Cavendish banana. Apical meristem aseptically removed from rhizomes was cut with 7-12 vertical incision and placed on a modified MS medium. Within one month a clustur of shoot formed which was separated and transferred individually to fresh medium. After three months plantlets with well developed shoot and roots were transplanted to soil.

Brower and Frazer (1982) regenerated plantlets by culturing growing point comprising of meristem and few leaf primordra of William banana in MS basal medium with different hormone concentrations.

Mante and Tepper (1983) cultured apical meristem slices in vitro in light on medium containing MS mineral salts supplemented with 100mg/l inositol, tyrosine, and ascorbic acid each, citric acid (50 mg/l), 2 mg/l cysteine, 0.4/l thiamine HCl, 3 per cent sucrose and 0.8 per cent agar. Shoot-tips were cultured on media containing 3-5 mg/l BAP, 0.1 mg/l IBA, 2-10 mg/l NAA and 160 – 200 mg/l adenine sulphate. Plantlets generated roots on media without adenine sulphate but, containing either NAA (2-10mg/l) or IBA (0.1 mg/l).

Doreswamy et al. (1983) reported the successful tissue culture propagation of banana through apical meristem of cultivar ‘Robusta’. They isolated the apical shoot buds and axillary buds from small suckers and cultured in the modified MS medium containing sucrose (2 %) and various growth regulators.
Cronauer and Krikorian (1984a) established rapidly multiplying cultures of desert banana clone (Philippine Lacatan and Granade naine) and plantain clones (Saba and Pelipita) from isolated shoot-tips on a modified MS medium supplemented with 1 mg/l thiamine HCl, 100 mg/l inositol, sucrose (1%) and 5 mg/l BAP. The growth rate of these cultivars, expressed as increase in fresh weight over a four weeks period was assessed. Rooted plantlets were produced using auxins NAA, IBA and IAA at 1 mg/l each with low levels of activated charcoal (0.025%). They also reported (1984b) that apices cultured in semisolid media produced single shoot while apices placed in liquid media, multiple shoot clusters were induced by longitudinally splitting the shoot through the apex. Further, regeneration of plantlets from these multiple buds was achieved by subculturing in a medium with low cytokinin.

Damasco and Barba (1985) reported that Musa spp cv Saba (BBB) formed multiple shoots on MS medium supplemented with 10 mg/l BAP. Subculture of shoot-tips to fresh medium of the same composition increased the number of shoots produced with each subculture. But Jarret et al. (1985) propagated shoot tips of ‘Saba’ and ‘Pelipita’ clones on a modified MS medium supplemented with BAP in combination with IAA. Multiple shooting was initiated by splitting shoot-tips along their longitudinal axis and re-culturing the individual pieces to MS basal medium supplemented with 5 mg/l BAP.
Vuylsteke and De Langhe (1985) reported high proliferative growth of adventitious buds of banana in modified MS medium supplemented with high cytokinin content (10μM BAP). Regeneration of plantlets from these multiple buds was achieved by subculturing on medium with low cytokinin content (1μM BAP) Varietal differences in the proliferation rates were grouped according to genome type - the AAB and ABB genome showed the highest multiplication potential.

Bhagyalaxmi and Singh (1995) cultured meristem explants of 3 cultivars of banana 'Cavendish', 'Bluggoe' and 'silk' on modified MS medium supplemented with 8.0μM BAP and 0.08μM IBA, in liquid and solid media. They found that shoot multiplication rate was enhanced when cultured in liquid media.

Mateille and Foncele (1988) described an improved micropropagation method for the Poyo banana. Main apices of sucker buds and lateral buds were stimulated by a high concentration of BAP (22.5μM) in the absence of auxin. After transplantation shoot proliferation was achieved with the same concentration of cytokinin. Shoot elongation and rooting were achieved on a medium containing no phytohormone and 10g/l sucrose. Rooting was improved (97.9%) when the bottom of the culture tubes with medium were set in darkness.

When the shoot-tip cultures of banana were transferred under minimal growth conditions the growth was stopped. This technique could be used as an in vitro approach to the conservation of Musa germplasm.

2.2.2 Regeneration from other explants

Cronauer et al. (1987) reported adventitious shoot production from calloid cultures of banana. Media containing MS basal, p-chlorophenoxyacetic acid (5.0mg/l) and kinetin produced a compact calloid mass. Transfer of calloid mass to MS basal supplemented with 1mg/l NAA led to the production of rooted plantlets (Cronauer et al.1988). Plant regeneration via somatic embryogenesis in the seeded diploid banana *Musa ornate* Roxb. was also obtained.

Determinate floral buds of plantain (*Musa ABB*) was used as explants and cultured on MS medium supplemented with 5mg/l BAP. Multiple shoots were regenerated and plantlets were produced (Cronauer et al. 1987). Balakrishnamurthy and Rangasamy (1983) regenerated banana plantlets from *in vitro* cultures of floral apices. Doreswamy and Sahilram (1989) micropropagated banana from male floral apices. Ganapati et al. (1992) regenerated banana plants from encapsulated shoot-tips.

Megia et al. 1993 regenerated plants from cultured protoplast of the cooking banana cv. Bluggoe (*Musa, spp, ABB group*).

Navarro et al. (1997) regenerated plants from embryogenic cultures of a diploid and triploid Cavendish banana. Lee et al. (1997) studied histology of somatic embryo initiation and organogenesis from rhizome explants of *Musa spp.cv 'Grand Nain'*. 
2.3.0 Culture Media

The successful establishment and growth of plant cells in vitro generally is determined by the nature of the explant and the composition of the medium (White 1951). White's medium (White 1943, 1963) in its modified forms continued to be extensively used until about 1965 for the growth of excised roots, embryos, callus tissues, floral organs etc. A systematic study of mineral requirements resulted in many media formulations that improved growth over that of Gautheret's (1939) or White's (1943, 1963). Major breakthrough in chemically defined medium was made by Murashige and Skoog (1962) which determined the mineral nutrient requirements. Vasil and Hildebrandt (1966) compared the growth of callus tissues of a wide variety of plant tissues grown in MS medium and other high salt formulations and showed that optimal increases in fresh weight, dry weight and chlorophyll development occurred in the MS medium. Several other media including that of B5 (Gamborg et al. 1968) and Schenk and Hildebrandt (1972) were later developed by substantially increasing the level of inorganic salts.

2.3.1 Inorganic Nutrients

In general, the tissue culture media contain 16 essential elements for plant growth. The macronutrients include six major elements; Nitrogen (N), Phosphate (P), Potassium (K), Calcium (Ca), Magnesium (Mg) and Sulphur (S) which are present as salts that constitute various media. These are essential for plant cell and tissue growth. Nitrogen is mostly provided in two forms as nitrate and as ammonium compounds. In most media, iron may be chelated as ferric sodium ethylene-diamine tetra acetate (Fe-EDTA). The micronutrients essential for plant cell and tissue growth are Manganese (Mn), Zinc (Zn), Boron (B), Copper (Cu) and Molybdenum (Mo). Certain media are enriched with Cobalt (Co), Iodine (I), Sodium (Na) and Chloride (Cl) but specific role of these elements for cultured cells and tissues have not been established (Torres 1989).

MS is considered to be the most successful medium for culture in most of the species (Santos et al. 1970, Engvild 1973, Smith and Gould 1989, Carrier and Cosentino 1990, Singh et al. 1993, Muthukumar et al. 1999), when compared with other media. But in a few species B5 medium has proved better supplement for regeneration (Neelam et al. 1986, Hazarika and Sarma 1996, Anand and Bansal 2002) or callusing (Thulaseedharan and Vaidyanathan 1990). Both MS and B5 media along with Vacin and Went and Knudson C (Knudson L. 1946) have been successfully used (Roy 1994, Bordoloi & Sarma 1992, 1997, Baruah and Sarma 1992, Hazarika and Sarma 1995, Kaur and Sarma 1997a; Sarma and Sarma 1997a,b; Roy and Sarma

The inorganic salt formulations can vary (Murashige 1973; Huang & Murashige 1977; Gamborg et al. 1976; George et al. 1984). The distinguishing feature of the MS inorganic salts is their high content of nitrate, potassium, and ammonium in comparison to other salt formulations.

2.3.2 Organic Nitrogenous supplement.

In culture, plant cells receive organic nitrogen in the form of amino acid as an immediate available source of nitrogen, which is easily and rapidly taken up by the cultured cells than inorganic nitrogen (Tom et al. 1981).

Amino acids and amines can be very important in morphogenesis. All L-forms of amino-acids are the natural forms detected by the plant. L-tyrosine can contribute to shoot initiation (Skoog and Miller 1957), L-arginine can facilitate rooting, and L-serine can be used in microspore cultures to obtain haploid embryos. Amides, such as L-glutamine and L-asparagine sometimes significantly enhance somatic embryogenesis. Some amino acids and amides viz. L-asparagine, L-glutamic acid, L-glutamine and L-arginine, traces of L-methionine give positive results (Robert and Baba 1978, Miller and Roberts 1984). Addition of amino acids to both B5 and MS media enhances efficiency of development of shoot (Mahalaxmi et al. 2003). Increased frequency of plant regeneration of barley in medium supplemented with alanine, asparagines and glutamine was observed by Zhu et al., (1990). That proline and glutamine enhanced adventitious shoot
induction in *Phyllanthus* was reported by Rajasubramanian and Sirothi (1994). Inclusion of some amino acids in culture medium has been shown to stimulate embryogenesis (Rao *et al.* 1995, Claparol *et al.* 1993).

Casein hydrolysate, an enzymatic digest of milk protein was a common ingredient in many early media formulations as it provided a mixture of amino acids to enhance tissue response. The role of CH in enhancing orchid seed germination and subsequent growth of protocorms leading to regeneration of plantlets was reported (Roy 1994, Bordoloi & Sarma 1992, 1997, Baruah and Sarma 1992, Hazarika and Sarma 1995, Kaur and Sarma 1997a; Sarma and Sarma 1997a,b; Roy and Sarma 1992a,b,1993; Sarma and Roy 1992-93, 1996; Devi 2004, Sarma and Devi 2006).

2.3.3 Coconut Water (CW)

The concentration of CW normally ranges from 10-30 per cent (Morel 1974, Goh 1982, Sagawa and Kunasaki 1984). The growth promotory effect of CW has been attributed to its PGR content and the most important PGRs are the cytokinins (Letham 1974, van Staden and Drewes 1975) whereas auxin and gibberellins - like constituents have also been reported (Dix and van Staden, 1982). The stimulatory effect of CW (15 per cent) has also been reported in *Musa sp* (Doreswamy *et al.* 1983).

van Overbeek *et al.* (1942) suggested the presence of a certain 'embryo factor' in the liquid endosperm of coconut water. Subsequently it became one of the most commonly used medium supplements. The 'embryo factor' of CW is heat resistant and can be sterilized either by filtration or by
autoclaving. Hu and Wang (1983) reported that in their laboratory they usually used 15 per cent (v/v) CW in culture medium.

Coconut water (CW), which is the liquid endosperm from *Cocos nucifera* L. induces division of the otherwise non-dividing cells (Goh *et al.* 1973, George and Sherrington, 1984).

### 2.4.0 Plant Growth Regulators (PGRs)

Plant growth regulators are organic compounds other than nutrients, which in small amounts promote, inhibit or otherwise modify physiological processes in plants (van Overbeek *et al.* 1954). The exogenous requirements for PGRs depend on their endogenous level in the plant growth system, and it varies with the tissue, plant type, and phase of the plant growth (Bhojwani and Razdan 1966). Many of these regulators including auxins, cytokinins, gibberellins, ethylene, abscissic acid etc. induce a variety of growth and development processes both *in vivo* and *in vitro* (Weyers and Peterson 2001).

There are reports to suggest that cells respond to culture medium and regenerate plants without the influence of exogenous plant growth regulators. For example, excised embryos of *Citrus sinensis* (Vardi *et al.* 1975) and *Ilex aquifolium* (Hu *et al.* 1978) have developed somatic embryos on cultured anthers (Nitsch and Nitsch 1969). However for the induction of callus and organogenesis and also for direct multiplication from cultured cells, tissue and organs it is just unthinkable to proceed for *in vitro* multiplication without PGRs.
2.4.1 Auxins

The term auxin was first used by a Dutch botanist, FW Went in 1926 for Indole-3-acetic acid (IAA). The auxins most commonly employed for tissue cultures are IAA, IBA, NAA and 2,4-D. Tissues from different varieties of the same species may show different auxin requirements.

2.4.1 (a) Indole 3-Acetic Acid (IAA)

Indole-3-acetic acid (IAA) was first identified in human urine (Kögl et al. 1934). The status of Indole-acetic-acid (IAA) as the most universal natural auxin in higher plants have been determined by the most sophisticated methods (Bentley 1958 and Shantz 1966). Working with leaf disks of *Phaseolus vulgaris* Sarma (1971) observed that IAA even at a concentration of $10^{-5}$ M was inhibitory, while at $10^{-6}$ a marked stimulation was caused. Sarma (1971) also reported expansion growth of leaf disks of *Phaseolus vulgaris*. Sarma and Borah (1972,74) reported that IAA stimulated extension growth of bean hypocotyl segments. At high concentration auxin does not allow lasting tissue growth on sunflower tissue culture (Henderson 1954).

In Chickpea (*Cicer arietinum* L) callusing was induced on both B5 and MS media supplemented with different combinations and concentration of auxins and cytokinins, but shoot generation was achieved only in B5 medium supplemented with 4.0mg/l IAA and 0.5mg/l BAP after serial subculture of callus on media with increasing concentration of IAA and constant concentration of BAP (Roy et al. 2001).
Sharma (1987) reported increased chlorophyll synthesis in *Rhaphanus* leaves by application of IAA. IAA enhanced synthesis of chlorophylls was coupled with an increase in thylakoid formation as a result of which the number of photosynthetic centres increased. Wightman and Lichty (1982) recorded the common occurrence of IAA and phenoxy-acetic acid in the young shoots of several higher plants.

There are some reports to establish that IAA can induce the synthesis of new RNA and protein in a variety of plant tissues. IAA induced RNA and protein synthesis in *Rhoea* leaves and bean endocarps (Sacher 1967), yeast cell (Shimoda *et al.* 1967), green pea stem sections (De Hertogh *et al.* 1965), oat coleoptile sections (Masuda *et al.* 1967) and excised soybean hypocotyls tissue (Key and Shannon 1964).

2.4.1 (b) Indole–3 butyric Acid (IBA)

Various workers observed superior action of IBA on root promotion over other growth regulators (Singh and Sharma 1954, Dikshit 1956, Fontanaza and Rugini 1981). In auxenic culture of pomegranate stem cutting IBA proved to be better than IAA for the induction of rooting (Ghosh *et al.* 1988).

Wilkins and Dodds (1983) observed IBA (5mg/l) to be the best among growth regulators on shoot extension and proliferation and leaf and callus induction of shoot cultures of semi dwarf cherry root stock and found shoot extension. Koblitz *et al.* (1983) recorded best results on MS medium containing IBA and arginine in cultured shoot tips of *Chinchona*. In shoot tip culture of Castanea *sativa* (Rodrigue 1982) and cotyledon culture of *Glycine*
max, IBA alone induced rooting but along with BA produced shoot proliferation.

The high IBA:BA ratio induced callus, however a low ratio induced shoots from this callus from leaf explants of *Ipomea batatas* (Bordoloi and Sarma 1992). That IBA induces rooting on *in vitro* produced plantlets was reported by Standardi and Romani (1990), Attfield and Evans (1991) and Evaldson (1985). Cossio (1981) suggested that MS medium containing 1mg/l BA and 0.1mg/l IBA is the best for shoot multiplication of *Aeschynanthus lobiana* and the medium with 3 mg/l BA and 1 mg/l IBA is the best for explants establishment. He obtained 20,000 plants within a year from a single *A. lobiana* shoot tip explants using this medium. Chopra (1957) in his *in vitro* culture of various explants of *Althaea rosea* L. observed that IBA at 20mg/l proved to be the best of the various growth regulators used.

2.4.1 (c) α Naphthalene Acetic Acid (NAA)

NAA stimulates growth of shoot and root system and proliferation of tissues, but on long term treatment it inhibits root elongation. The auxin activity of NAA, a synthetic growth regulator varies considerably. Gautheret (1942) and Morel (1948) examined the efficiency of NAA, in culture media. Stem segments of papaya seedlings proliferated only when NAA was used as an auxin in MS and LS medium (Arora and Singh 1978). In *Asparagus* the most successful growth regulator for shoot proliferation was NAA at a concentration of 0.05 ppm (Zou and Mang 1981). Bhansali *et al.* (1978) showed that higher NAA concentration (1-10 ppm) were inhibitory to callus
growth. On the other hand, low concentrations of NAA (0.1-0.5 mg/l) induced shoot proliferation in cherry root stocks (Wilkins and Dodds 1983). Response of NAA also varies with species viz. increasing concentrations of NAA (2 mg/l and 3 mg/l) show positive result in shoot regeneration from leaf explants of *Indian spinach* (Mitra and Mukherjee 2001). Similarly in *Lilium mackliniae*, a rare endemic endangered plant produced best bulblets and leaf growth with different type of explants in MS medium supplemented with NAA alone (0.5 µM) than in combination with any cytokinin (Mao et al. 2002). Lane and Mc Dougald (1982) also showed that best rooting occurred at 1.0 or 3 µM NAA on shoot culture of apple.

Rapid proliferation of axillary buds of trailing black berry and thornless young berry was achieved on a modified MS medium containing NAA (0.1 mg/l) and BA (2 mg/l) (Skirvin *et al.* 1981). MS medium containing NAA (0.1 mg/l) and BA (2 mg/l) induced shoot proliferation from shoot tips of peach, cherry and apricot (Skirvin *et al.* 1981). Axillary buds elongated most rapidly from nodes of *Paulownia tomentosa* on a medium containing BA (1 mg/l) and NAA (0.1 mg/l) (Burger *et al.* 1985).

2.4.1 (d) 2,4-Dichlorophenoxy – Acetic Acid (2,4-D)

The most effective herbicide for callus proliferation is 2,4- dichlorophenoxy acetic acid (2,4-D). This herbicide is a powerful suppressant of organogenesis and should not be used in experiment involving root and shoot initiation (Gamborg *et al.* 1976).
Combination of 2,4-D and coconut milk had a remarkable effect on proliferation of carrot and potato tissue (Caplin and Steward 1948, Steward and Caplin 1951, 1952). It is the most widely utilized callusing hormone. Many workers had used it for the development of different parts of plants (Pershina and Isaeva 1982, Wiegel and Hughes 1983, Branton and Blackers 1983). In Sorghum 2 mg/L of 2,4-D induced callusing maintained in 1 mg/L of 2,4-D and regenerated in hormone free medium (Ture et al. 1995). 2,4-D was used for callus induction in Alternanthera sessilis (Boro et al. 1998). In six different cultivars of rice studied by Sankhdhar et al (2001) 2,4-D proved to be very effective in callus formation which on transfer to regenerative medium showed good regeneration capacity. 2,4-D is the best auxin for callus formation in Calamus tenuis (Sett et al. 2002).

Trolinder and Goodin (1988) observed that seedling explants of Gossypium formed somatic embryos in 100 per cent cultures when treated with 0.1 mg 2,4-D plus 0.5 mg/L kinetin. This result was better than kinetin plus NAA treatment. However in leaf explant they observed 2,4-D (0.1 mg/L) plus 1.0 mg/L (2-isopentyl) adenine as best supplements.

2,4-D with BA or kinetin resulted in callus formation in Digitalis thapsi and the seedling derived callus was maintained for two years (Herrera et al. 1990). In Bauhinia malabarica Roxb. root culture, BA alone was not sufficient for regeneration and required an auxin for bud induction and preferred 2,4-D over NAA. 2,4-D (2 mg/L) with BAP (1 mg/L) in MS
medium showed 55-60 per cent regeneration (Dasgupta and Bhattacharya 1995).

Finer (1987) observed direct somatic embryogenesis from immature zygotic embryos on high sucrose containing medium in presence of 2,4-D. 2,4-D also induced somatic embryos on suspension culture of hypocotyls derived callus of sunflower (Prado and Berville 1990).

Shoot induction of *Datura metel* in only 2,4-D derived callus had been best in low concentration of 2,4-D (1mg/l) followed by 1.5 mg/l and 2 mg/l when combined with 2 mg/L BAP (Muthukumar *et al.* 2001). In *Acacia mangium* callus induction occurred in 2,4-D (9.05μM) from all types of callus (Xie and Hong 2001). Arau *et al.* (2001) reported negative effect of 2,4-D on 4 glossy and 1 nonglossy *Sorghum* lines.

### 2.4.2 Cytokinins

Cytokinins are mainly concerned with cell divisions and modification of apical dominance. They are applied for shoot differentiation in tissue culture. Addition of cytokinin promotes precocious axillary shoot development (Hussey 1976). Most commonly used cytokinins in tissue culture are kinetin (6-furfurylaminopurine) and BAP (6- benzylaminopurine or 6- benzyladenine). It may be interpreted that the response of a system to different cytokinins may be tissue specific (Arteca 1997) or the cytokinins can be mutually substituted.

#### 2.4.2 (a) Kinetin (KN)

Kinetin is probably not a natural component of plants. Miller and his co-workers (Miller *et al.* 1955) isolated an active cell-division factor from
autoclaved herring sperm DNA and identified it as 6-furfurylaminopurine and named it as ‘Kinetin’ (C_{10}H_{9}N_{5}O).

That kinetin stimulates cell division in plant tissue cultures was the first effect to be observed (Miller et al. 1956, Skoog and Miller 1957). Kinetin in the presence of IAA, induced proliferation of tobacco cells and also bud formation (Skoog and Miller 1957). A combination of kinetin with IAA evoked a high spate of cell division. Patau et al. (1957) suggested that auxins and cytokinins might affect distinctive mitotic steps, including the doubling of the DNA content of the nucleus.

Kinetin is reported to induce cell enlargement besides causing cell division. Many workers (Miller 1956, Powell et al. 1960, Sarma 1971) reported that treatment of leaf disks cut from etiolated leaves with kinetin caused significant cell enlargement.

Kinetin treatment caused an increase in fresh weight of the epicotyl, expansion of leaf and increase in the elongation of stems and petioles of bean (Miller 1956). Kinetin enhances chloroplast differentiation mainly in cases in which it is not required for culture growth (Neumann and Rafaat 1973, Seyer et al. 1975) because it can stimulate chloroplast replication and maturation without inducing cell division.

The effect of kinetin also varies with the type of the media used viz. *Adhatoda vasica* axillary bud culture in the combination of kinetin and NAA showed bud break in B_{5} medium while it was not in MS medium (Anand and Bansal 2002). Kinetin (10 μM) proved best for differentiating a
maximum of 15.25 ± 4.72 shoots per explant in 75 per cent in *Centella asiatica* (Agarwal and Subhan, 2003). Koshta and Bansal (2002) found axenically produced bud explants of *Rauvolfia serpentina* exhibited excellent *in vitro* response in the form of bud break on kinetin (0.5 mg/l). The best results for shoot multiplication of *Suaeda nudiflora* were recorded on MS medium supplemented with kinetin (0.2 mg/l) and BAP (1.0 mg/l) in axillary bud culture (Cherian and Reddy 2002).

2.4.2 (b) 6-benzyladenine (BA)

One of the most important synthetic cytokinins is BA (C₁₂H₁₁N₅), which is active in some assays than even the most active natural cytokinin. Multiple shoots regenerated from young strawberry plants cultured on MS medium containing BA (0.02–0.2 mg/l) (Lee and Park 1980). Burger *et al.* (1985) suggested that axillary buds elongate more rapidly on a medium containing 1 mg/l BA and 0.1 mg/l NAA. BA (5-20 mg/l) was necessary for callus and adventitious shoot formation but not for axillary shoot proliferation (Ripley and Preece 1986).

BA is the most frequently used cytokinin in commercial micropropagation (Thomas and Blakesley 1987) as it reduces more proliferation than zeatin or kinetin (Marino and Bertazza 1990). In *Mitragyna parvifolia* Korth. BA gave better results in comparison to kinetin in apical meristem culture on MS medium showing 8-10 plantlets per explant in 20-30 days. Gonga (1993) recorded multiple shoot formation from shoot tip cultures in *Sesamum indicum* L. with high BAP concentrations. In shoot tip culture,
maximum shoot proliferation was observed in the medium supplemented with 1 mg/l BA while in young phyllode cultures maximum shoot proliferation was observed.

Panaria et al. (2000) described a method for *in vitro* propogation of *Symononthus bancroftii*, a critically endangered shrub which contained BA in the medium. Ravishnakar et al. (2000) described a method for *in vitro* propagation of *Decalepis hamiltonii*, an endangered shrub through axillary bud culture. Axillary explants produced multishoots when cultured on MS medium supplemented with BA (2.5mg/l) (Tetyana and van Staden 2001).

Mukundan et al. (2002) reported maximum number of shoots on MS medium containing 1.5 and 1.0mg/l BAP for *Tylophora asthmatica* and *Uraria picta* respectively. In shoot tip culture of *Rauvolfia serpentina* bud break occurred both in BAP and kinetin but shoots produced in higher concentration of BAP (5mg/l) were healthier (Koshta and Bansal 2002).

Shoots were induced from rhizomes on basal MS medium containing 120 g/l sucrose and 5 g/l agar supplemented with 20 per cent (v/v) coconut water (CW) and BA at concentration from 0.5-5 mg/l (Nguyen et al. 2005). In *Jatropha curcas* L, combination of BAP (2.0 mg/l) and IAA (0.5 mg/l) proved optimal for maximum shoot proliferation (Rajore and Batra 2005).

**2.5.0 Carbohydrates**

In plants, carbohydrates act as a substrate for respiration, play a role in the synthetic pathways of many macromolecules. In addition they may also
control developmental process (Gibson 2000; Smeekens 2000). Sucrose proved to be best source of carbohydrate; because it is the most common carbohydrate in the phloem sap of angiosperms (Zimmermann and Zeigler 1975) and is also required for differentiation of xylem and phloem element in the cultured cells (Aloni 1980).

Gautheret (1945) worked on carbohydrate nutrition of plant tissue culture and concluded that sucrose served as the best carbon source followed by glucose, maltose and rafinose, fructose and galactose came next than mannose and less efficient was lactose. Sarma (1971) screened disaccharides and monosaccharides and found sucrose to be the best followed by glucose and fructose. However in some species sucrose has been reported to be inhibitory to germination, e.g. in Bletilla striata (Smith 1973). The exact requirement of sucrose in cultures of orchid proves effective at concentration ranging from 0.5 to 3 per cent (Arditti and Ernst 1984; Chia et al. 1988; Butcher and Marlow 1989). Higher doses of sucrose have been reported to be detrimental to germination and protocorm development of orchid seeds (Rubluo et al. 1989).

The choice and concentration of sugar to be used depend mainly on plant tissue to be cultured viz. Xylogenesis influences difference in carbohydrate (Roberts 1976, Robert and Baba 1982). Kaur (1999) reported 3 per cent sucrose as an ideal concentration for \textit{in vitro} germination of Arundina gramnifolia seeds. Most of the workers in our laboratory used 3 per cent sucrose in MS or B\textsubscript{5} media for micropropagation of orchid or

Myo-inositol is a carbohydrate and generally added in stock solutions of vitamins. Its presence in small quantities stimulate cell growth in most species (Murashige 1974). It is believed to be broken down into ascorbic acid and pectin and is incorporated into phosphoinositides and phospatidylinositol, which play a role in cell divisions (Torres 1989).

2.6.0 Vitamins

Vitamins have catalytic functions in various metabolic processes. In vitro grown cells and tissues synthesize some essential determines but do not reach the optimal level. Hence it is necessary to supplement the medium with required vitamins to obtain the desired objectives. The vitamin considered important for plant cells is thiamine (B₁). Other vitamins, nicotinic acid (B₃) and pyridoxine (B₆) are usually added to culture media (Bonner 1940, Gautheret 1942, White 1943). But Torres (1989) demonstrated that these are not essential for cell growth in many species. Murashige (1974) had clearly demonstrated thiamine as the most critical vitamin in plant tissue culture research. The view that thiamine is an essential vitamin for in vitro culture was also put forth by Ohira et al. (1976). Most of the workers add vitamin stock solutions to the medium before autoclaving. However, for specific studies on vitamins, they should be filter sterilized (Ten Ham 1971).
2.7.0 Antibiotics

Because of excessive contamination problems with certain plant explants, many workers have incorporated fungicides and bactericides in the culture medium (Thurston et al. 1979). Generally, these additions have not been very useful because they can be toxic to the explants and the contaminant can reappear as soon as the fungicides or bactericides are removed. Commonly used antibiotics are timentin, carbenicillin (500 mg/l), cefataxime (300 μg/ml) etc; and their solution should be made fresh for each application.

2.8.0 Antioxidant

Antioxidants are sometimes added when there is excessive browning of the explants which may cause death of the explants due to the formation of quinonic compounds. Examples of antioxidants are citric acid, ascorbic acid, pyrogallol and phloroglucinol and borine serum. Sometimes when there is excessive browning of the medium and explant, adsorbents are used. Two adsorbents in use are polyvinyl pyrrolidone (PVP) and activated charcoal in percentage of 0.1-0.3 (Mohamed-Yasseen et al. 1995, Wann et al. 1997). Musa is one of the common species where antioxidants need to be used. Ascorbic acid which may be used with other organic acids is useful as an antioxidant to alleviate tissue browning (Reyholds and Murashige 1979). Antioxidants are much more effective when added in liquid medium of static culture than when added to solid medium (Hu and Wang 1983).
2.9.0 Solidifying Agents (Agar)

Many tissue culture experiments are conducted on some type of stationary support and a gelling agent is most commonly used. Agar is the most widely used solidifying or gelling agent, which does not react with the other constituents of the medium and is not digested by the enzymes of plant cells. Normally 0.5-1 per cent is used in the media to form a firm gel at the pH typical for plant cell tissue and organ culture media (Torres 1989). The type of agar used to gell the medium can affect the response of the experiments (Griffis et al. 1983, Debergh 1983). Lal and Singh (1995) suggested that higher concentration favours multiple shoots and increase in root density. Selby et al. (1989) reported that decreased agar and low \( p^H \) favour rooting.

2.10.0 \( p^H \) of the Medium

The \( p^H \) of plant tissue culture media is generally adjusted in a slightly acidic range (\( p^H \) 5.5 to 5.8). Below 5.5, the agar will not gel properly and above 5.8, the gel may be too firm (Murashige 1973). Media \( p^H \) generally drops by 0.6 to 1.3 units after autoclaving (Sarma et al. 1997). Cultures of some plant tissues cause a \( p^H \) drop over time that is attributed to the production of organic acids or nitrogen utilization. Owen et al. (1991) examined media \( p^H \) as influenced by inorganic salts, carbohydrate source, gelling agent, activated charcoal, and medium storage method. All of these factors influence the \( p^H \). Adjustment of \( p^H \) should be done before adding the agar.
2.11.0 Activated Charcoal

The addition of activated charcoal to culture media is reported to stimulate growth and differentiation. Activated charcoal attributes to three factors: (i) Darkening of medium (ii) adsorption of inhibitory compounds and (iii) adsorption of plant growth hormones from the medium. Activated charcoal is generally acid-washed and neutralized before its addition at 0.5 per cent to 3 per cent to the culture medium. Fridborg and Erikson (1975) reported that activated charcoal promoted embryogenesis in callus culture by adsorbing inhibitory substances or metabolites released by the culture into the medium.

Some investigators included activated charcoal (0.1-0.25 per cent) for the induction of root (Hwang et al. 1984). Cronauer and Kirkorian (1984) and Jarret et al. (1985) found that it failed to promote root initiation.

2.12.0 Environmental Factors

The basic pattern of growth in culture is determined by the heredity of the plant species and the detailed expression of that growth is influenced by environmental factors chiefly by temperature and light. Plant cells in vitro conditions require proper aeration which is also an essential requirement for suspension culture (Gamborg et al. 1981).

2.12.01 Light

Among the various components of the physical environment, light is the most important external factor for growth and development of plants. High light intensity often causes losses of chlorophyll, yellowing or burning of leaves. On the other hand insufficient light promotes spindly and succulent growth.
Low light intensity in the order of 1,000-5,000 lux with a 16 hr/day provided by fluorescent tubes is generally adequate for shoot multiplication (Hussey 1986). A definite photoperiod (16 hr light, 8 hr dark) in contrast to continuous light or darkness was essential for shoot regeneration in Pelargonium zonale (Pillai and Hilebrandt 1964).

Low light intensity, while temperature is below 25°C enhanced bud formation in tobacco culture; however high intensity light and temperature inhibited it (Skoog 1944). Low intensity white light acted synergistically to the rhizogenic effect of auxin (Gautheret 1971). Optimum light intensity of 1000-lux was recommended for Asparagus, Gerbera, Saxifraga and Bromeliads culture (Murashige 1974). Kasperbauer and Reinert (1967) found that growth response to red light was reversed by far-red on stem culture of Nicotiana tabacum. Light intensity of 1000-5000 lux is generally adequate for shoot multiplication (Hussey 1990). Low frequency light resulted in less embryonic abnormalities in Caraway and Carrot (Ammirato 1974, 1983).

Even if rooting is induced in dark a pre-transplant exposure of plantlets to high light intensity (3,000-10,000 lux) favours better survival and early growth of the potted plants (Murashige 1974).

2.12.02 Temperature

It is one of the most important external factors, which influences the rate of growth of plants. Normally the temperature of culture room during incubation period is maintained between 15 to 30°C with a fluctuation of less than 0.5°C.
A wide range of temperature is required for specific experiments (Torres 1989). Murashige (1977) reported that warm temperature about 27° C are optimum for tropical genera and cool temperature (about 20°C) favour in vitro development of alpine plants. Workers from various tissue culture laboratories suggested 25-28°C range of temperature to support better growth for all types of plant tissues.

Roca (1984) observed that tissues of tropical plants like Musa sp. grow best in higher temperature than those appropriate for temperate species. Temperature also markedly influenced the rate of regeneration and multiplication of regenerated plants (Koevary et al. 1978).