During past few years, there has been an increase interest for *in vitro* multiplications and germplasm conservation of rare, endangered, aromatic and medicinal plants. Mass propagation of plant species through *in vitro* culture is one of the best and most successful examples of commercial application of plant tissue culture technology. Recently, there has been much progress in this technology for some plant which has multiple economic values and become very attractive due to its energy resources.

Micropropagation is the practice of rapidly multiplying stock plant material to produce a large number of progeny plants, using modern plant tissue culture methods. It is used to multiply novel plants, such as those that have been genetically modified or bred through conventional plant breeding methods. It is also used to provide a sufficient number of plantlets for planting from a stock plant which does not produce seeds, or does not respond well to vegetative reproduction. Micropropagation is a set of procedures that multiply plants in tissue culture with minimal genetic and epigenetic variability. Micropropagation is one of the innovative methods of asexual propagation, which proved to be effective for in vitro propagation of medicinal and endangered plants (Pattnaik & Chand, 1996). Clonal propagation through tissue culture has the potential to provide high multiplication rates of uniform genotypes, resulting in short term gains (Gupta et al., 1993). Cloning allows for the immediate and total capture of genetic gain. Clonal propagation through tissue culture popularly called micropropagation can be achieved in short time and space. Micropropagation technique would play an important role in mass propagation of elite genotypes, conservation of germplasm, genetic improvement of plants and production of pharmaceuticals yet it not replaced traditional methods of propagation, but has found its own niche in areas where it is clearly superior. Micropropagation involves following methods:

- 1. Establishment.
- 3. Rooting.
- 4. Hardening.
Establishment:

Micropropagation begins with the selection of plant material to be propagated. Clean stock materials that are free of viruses and fungi are important in the production of the healthiest plants. Once the plant material is chosen for culture, the collection of explant(s) begins and is dependent on the type of tissue to be used; including stem tips, anthers, petals, pollen and others plant tissues. The explant material is then surface sterilized, usually in multiple courses of bleach and alcohol washes and finally rinsed in sterilized water. This small portion of plant tissue, sometimes only a single cell, is placed on a growth medium, typically containing sucrose as an energy source and one or more plant growth regulators (plant hormones). Usually the medium is thickened with agar to create a gel which supports the explant during growth. Some plants are easily grown on simple media but others require more complicated media for successful growth; plant tissue grows and differentiates into new tissues depending on the medium. For example, media containing cytokinins are used to create branched shoots from plant buds and it happens in a vegetative form.

Multiplication:

Multiplication is the taking of tissue samples produced during the first stage and increasing their number. Following the successful introduction and growth of plant tissue, the establishment stage is followed by multiplication. Through repeated cycles of this process, a single explant sample may be increased from one to hundreds or thousands of plants. Depending on the type of tissue grown, multiplication can involve different methods and media. If the plant material grown is callus tissue, it can be placed in a blender and cut into smaller pieces and recultured on the same type of culture medium to grow more callus tissue. If the tissue is grown as small plants called plantlets, hormones are often added that cause the plantlets to produce many small offshoots that can be removed and recultured.

Rooting:

This stage involves treating the plantlets/shoots produced to encourage root growth. It is performed in vitro, or in a sterile test tube environment. Haening refers to the preparation of the plants for a natural growth environment. Until this stage, the plantlets have been grown in ideal conditions, designed to encourage rapid growth.
Hardening:

In the final stage of plant micropropagation, the plantlets are removed from the plant media and transferred to soil or (more commonly) potting compost for continued growth by conventional methods. Hardening typically involves slowly weaning the plantlets from a high-humidity, low light, warm environment to what would be considered a normal growth environment.

Advantages:

Micropropagation has a number of advantages over traditional plant propagation techniques:

- The main advantage of micropropagation is the production of many plants that are clones of each other.

- Micropropagation can be used to produce disease-free plants.

- Micropropagation produces rooted plantlets ready for growth, saving time for the grower when seeds or cuttings are slow to establish or grow.

- It can have an extraordinarily high fecundity rate, producing thousands of propagules while conventional techniques might only produce a fraction of this number.

- It is the only viable method of regenerating genetically modified cells or cells after protoplast fusion.

- It is useful in multiplying plants which produce seeds in uneconomical amounts, or when plants are sterile and do not produce viable seeds or when seed can't be stored (recalcitrant seeds).

- Micropropagation often produces more robust plants, leading to accelerated growth compared to similar plants produced by conventional methods - like seeds or cuttings.

- Some plants with very small seeds, including most orchids, are most reliably grown from seed in sterile culture.

- Some woody plants that are difficult-to-propagate by cuttings, layers or grafts can be micro propagated

- A greater number of plants can be produced per sq.
In the past, *in vitro* propagation of various plant species has been done successfully. Several types of DNA markers have been developed and used in plant research. RAPD markers are widely used for assessment of genetic variation within and between populations of plant species, germplasm assessment, characterization of clones, contamination of clones, testing genetic fidelity of tissue culture plants and detection of somaclonal variation in regenerated plants. This chapter briefly reviews the work done on micropropagation of plant species and effects of various factors on *in vitro* propagation of plants. It also gives a concise account of the work done on various aspects of RAPD analysis of some plant species.

### 2.1 Establishment:

Usually the juvenile explants are obtained from aseptically grown seedling. However, the nursery plants can also be used for this purpose. Mature explants are obtained from field grew trees of different age and height. The source of rejuvenated explants differs according to the technique employed for rejuvenation. The explants obtained from the field grew plants are sterilized and placed on culture medium for outgrowth of shoot buds. There are several factors that influence the establishment of explants.

The main factors influencing this stage are:

- Explants establishment
- Initiation of culture
- Surface sterilization
- Basal medium
- Plant growth regulators
- Phenolic exudation

#### 2.1.1 Explants Establishment:

The field-grown plants are sometimes unsuitable to initiate cultures. This stage involved in preparation of mother plants and type of explant should be taken so that improved quality explants can be obtained for establishment of culture. This can be accomplished by exposing the stock plants to suitable light, temperature, growth regulators, growing in glass house etc. Cultures are generally initiated from sterile
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pieces of a whole plant. These pieces are termed ‘explants’, and may consist of pieces of organs, such as leaves or roots, or may be specific cell types, such as pollen or endosperm. Many features of the explants are known to affect the efficiency of culture initiation. Generally, younger, more rapidly growing tissue (or tissue at an early stage of development) is most effective. To minimize the rate of infection and to reduce the chance of contamination during explants establishment, the mother plants are grown under suitable environmental conditions. Softwood shoots forced from various lengths of large stem segments that were removed from intact trees and shrubs, can be used as explants (Aftab & Preece, 2007). The cotyledonary nodes served as explants source for in vitro propagation of Crotalaria verrucosa, a medicinal herb (Hussain et al., 2008), Acasia sinuata (Vengadesan et al., 2002), Prunus persica (Mante et al., 1989), Corchorus capsularis (Naheer et al., 2003; Saha et al., 1999), Vigna radiata (Gulati et al., 1992). Siddique & Anis, 2009 used mature fruit of Balanites aegyptiace for in vitro propagation. Sharma et al., 2007 maintained the plants of Bacopa monnieri in the net house before using them as explants source for in vitro propagation. Debnath, 2004 reported green house grown plants to develop protocol for micropropagation of dwarf Rubus pubescens through in vitro axillary shoot proliferation. Rathore et al., 2008 germinated seeds of Terminalia bellerica aseptically on to water agar (0.8%) to obtain nodal segments of seedlings. They treated the seeds of T. bellerica with 0.15% HgCl₂ for 15 minutes, washed with sterile distilled water and inoculated aseptically on to water agar (0.8%) for germination. Aseptically-grown seedlings have been taken as explants source for in vitro propagation of Calophyllum inophyllum (Thengane et al., 2006). Seeds taken from mature fruits were treated with savlon, PVP, bavistin, ethyl alcohol and finally with mercuric chloride, each time washing with distilled water was germinated in different media.

2.1.2 Initiation of Culture:

For initiation of culture, development stage of explants is an important factor for which selection of an explant in the optimal physiological and developmental state is the most important parameter governing the success of shoot formation. The age of the stock plant, the physiological age of the explants and its development stage, as well as its size can also determine the success of a procedure. Seedling explants are more responsive compared to the mature plant derived explants. Younger tissue, such as terminal or axillary shoot apices or tips of adventitious shoot regenerate better than...
older and more mature tissue of the same plant. Frequency of sprouting is always higher in the buds taken from plant during its vegetative phase. Das & Pal, 2005 used nodal stem segment of *Bambusa balcooa* and *Bambusa tuda* with an axillary bud at the centre and internodes on either side were excised and used as explants after removal of the leaf segment. Different plant tissue viz., intermodal segments (Sujatha et al., 1995), shoot tip (Islam et al., 1993) and apical and axillary buds (Gupta et al., 1980, Rout & Das, 1993) have been taken as explants. Apical buds of 8–10 mm were excised from 5 days old *in vitro* seedlings were used as explants in *Psoralea corylifolia* (Baskaran & Jayabalans, 2008). Cotyledonary nodes explants were excised from 6-, 12-, 18-, and 24-d old in *Pterocarpus marsupium* seedlings (Husain et al., 2007). Axillary buds were used as explants from a 27-year-old mother plant of Cornusmas ‘Macrocarpa’ (Jaroslav, 2008). Axillary shoot proliferation from CNs of seedlings was well documented for several tree species such as *Acacia nelotica* (Dewan et al., 1992), *Sterculia urens* (Purohit & Dave, 1996), *Dalbergia sissoo* (Pradhan et al., 1998), *Acacia sinuata* (Vengadesan et al., 2002), and *Sesbania rostrata* (Jha et al., 2004). Dubey et al., 2004 taken apical buds of 3 to 5 year old plants and 30 to 32 year old trees of *Adina cordifolia* as explants source.

Nodal segments from 7 month old plants of *Jatropha curcas* (Datta et al., 2007), 2-year-old valuable multipurpose medicinal plant *Ocimum gratissimum* (Gopi et al., 2006), apical and nodal segments from 3-year-old green house grown important medicinal plant *Cordia verbenacea* (Lameira & Pinto, 2006) and young leaves of 40-year-old ornamental tree oleaster (*Elaeagnus angustifolia*) (Karami & Piri, 2009) were employed as explants for micropropagation. Sakila et al., 2007 used fresh nodes from mature plants of perennial, stoloniferous, commercial herb, strawberry (*Fragaria x ananassa*). Feyissa et al., 2005 developed an *in vitro* propagation method for *Hagenia abyssinica* using leaf explants from both *in vitro* seedlings and mature trees. The rate of regeneration was significantly different among explants of seedling origin and mature trees. Nodal segments from plants of different age have been employed to initiate shoot bud cultures. Sujatha et al., 2007 selected nodes and cotyledonary nodal segments of 8 day old seedling of *Cicer arietinum*. Rout et al., 2008 germinated semi mature seeds of *Acacia chundra* for 15 days *in vitro* to obtain shoot tips and nodal segments as explants source. Similarly, cotyledonary nodes from 15 days old *in vitro* cultured seedlings of *Sterculia urens* (Hussain et al.,
2007) and Crotalaria verrucosa (Hussian et al., 2008) served as explants source for micro propagation. The internode stem segments of 3-week-old medicinal crop Mentha x piperita were used for in vitro propagation (Wang et al., 2008). Oet et al., 2004 initiated cultures from axillary node explants of 30 day old seedling of Gymnema sylvestre.

It has been reported that the age of the tree and genotype affected the micropropagation of mature trees. In Sorbus aucuparia, Chalupa, 2002 reported that nodal plants from lower branches and epicormic shoot of mature trees of this species, exhibit high shoot multiplication coefficients when cultured on modified MS agar nutrient medium supplemented with cytokinin (BA and TDZ) and auxin (IBA). He also found that height and diameter increments of micropropagated trees originated from juvenile parts of mature trees were considerable and their dimensions after five years of growth were comparable with the dimensions of trees originated from seeds. It has also been reported that selection of explants, composition of nutrient media, concentration of phytohormones and methods used for micropropagation have significant effects on shoot multiplication rates, rooting and quality of produced trees (Chalupa, 2002) levels of morphogenesis than apical ends. Quraishi et al., 1997 reported that the cultures derived from terminal branches of 50 years old tree explants of Lagerstroemia parviflora died after third transfer, whereas micro shoots obtained from seedlings and basal sprouts of another 50 year -old tree explants showed 10% rooting on MS medium supplemented with 4.9 μM IBA. Apical and nodal segments of Cordia verbenacea approximately 30 to 40 mm long were harvested from three year old greenhouse grown plants (Lameira & Pinto, 2006). In Sterculia urens (Hussain et al., 2007a), fifteen days old seedling were used as the source of shoot tip, axillary bud and cotyledonary node explants for in vitro propagation. Khalafalla & Daffalla, 2008 employed cotyledonary node explants excised from 7 days-old in vitro raised seedling and nodal segment explants excised from twelve-months-old plant of Acacia senegal. Nodal buds were collected from 15 - 20 year old trees of Holarrhena antidysenterica for in vitro propagation (Mallikaarjuna & Rajendrudu, 2007).

2.1.3 Surface Sterilization:

Sterilization is an essential step before placing the explants on establishment medium. Chemical sterilization ensures removal of microbes from surface of the
explants without compromising viability. Various types of treatments have been used for the sterilization of the explants. Sterilizing agents such as calcium and sodium hypochlorite, hydrogen peroxide, bromine water, silver nitrate, mercuric chloride and antibiotics at various concentrations and treatment duration are useful to disinfect plant tissue (Bhojwani & Razdan, 1996). Sterilization of seeds avoids direct exposure of explants to sterilizing agents. Hassan et al., 2008 surface sterilized Eclipta alba, explants with 0.1 % mercuric chloride for 5 min. followed by five rinses with sterile double distilled water in front of a laminar air flow cabinet. Mohapatra & Rout, 2005 reported 0.1 % mercuric chloride solution for 15 min for surface sterilization of Geoderum purpureum explants. Chishimba et al., 2000 used 30% NaOCl to decontaminate Uapaca kirkiana seedling. Among the several chemicals, HgCl$_2$ is considered as a potent surface-sterilizing agent; however, its residual inhibitory effect is also greater than the others commonly used in plant tissue culture (Bhojwani & Razdan, 1996). Jaskani et al., 2008 surface sterilized nodal segments (0.5-0.8 cm), shoot tips (0.3 – 0.5 cm) and leaf disc (0.5 cm$^2$) explants by rinsing in tap water for 5 minutes, dipped in 70% ethanol plus 2 drops of Tween-20 for 5 minutes followed by 3 washings in autoclaved distilled water and then treated with chlorox (10%) for 10 or 15 minutes The Phaseolus vulgaris seeds were washed with running tap water and soaked for 4 min in 70% ethanol, disinfected with 20% (v/v) sodium hypochlorite with two drops of Tween-20 solution for 20 min and rinsed three times with sterile distilled water then the seeds were immersed in 0.01% Benomyl (Piscis, Costa Rica) solution for 4 minutes and rinsed three times with sterile distilled water (Arias et al., 2010). Yildirim & Turker, 2009 washed seeds of Filipendula ulmaria with an antibacterial soap, rinsed with distilled water, surface sterilized by shaking in 20% ethanol for 10 minutes, rinsed with sterile distilled water, dipped into 10% Domestos (5% v/v sodium hypochlorite) for 20 min and finally washed with sterile distilled water three times. Verma et al., 2008 surface sterilized healthy fruits of Trichodesma indicum with 0.2% HgCl$_2$ solution for 15 minutes, rinsed 4-5 times with autoclaved distilled water and extracted embryo explants from fruits. Datta et al., 2007 collected nodal explants (2-3 cm in length) from the seven-month-old donor plants of J.curcas, kept them for 3 h in a systemic fungicide, Bavistin (BASF India Ltd.) and surface-sterilized in 0.1% HgCl$_2$ (w/v) for 20–25 minutes followed by repeated washing (five times) with sterile distilled water.
2.1.4 Basal Medium:

One of the most important factors governing the growth and morphogenesis of plant tissues in culture is the composition of the culture medium. The basic nutrient requirements of cultured plant cells are very similar to those of whole plants. Plant tissue culture media are generally made up of some or all of the following components: macronutrients, micronutrients, vitamins, amino acids or other nitrogen supplements, sugar(s), other undefined organic supplements, solidifying agents or support systems, and growth regulators. Several media formulations are commonly used for the majority of all cell and tissue culture work. These media formulations include those described by the scientist White, Murashige & Skoog, Gamborg et. al., Schenk & Hilderbrandt, Nitsch & Nitsch and Lloyd & McCown. Murashige & Skoog’s (MS medium) (1962) Schenk & Hildebrand’s (SH medium) (1972), Lloyd & McCown’s, Woody plant Medium (WPM) (1980) and Gamborg’s (B-5) (1968) medium are all high in macronutrients, while the other media formulations contain considerably less of the macronutrients. There are more medium used for micropropagation of plants like Driver & Kuniyaki (DKW) (1984), Gresshoff & Doy (GD) medium (1972), Linsmaier & Skoog (LS) (1965), Heller’s medium (Vieitez et al., 1989) etc. But MS medium is more frequently used. Gantait et. al., 2011 accelerated in vitro propagation in Aloe vera L. Culture initiated with rhizomatous stem on Murashige and Skoog (MS) medium fortified with 0.5 mg l⁻¹ Naphthalene acetic acid and 1.5 mg l⁻¹ N6 Benzyaminopurine (BAP) which promoted earliest shoot induction. Maximum shoot multiplication was achieved in MS medium supplemented with 2.5 mgL⁻¹ BAP. The best in vitro rooting was observed in the MS medium with 0.5 mg L⁻¹ indole-3-acetic acid plus 2 gL⁻¹ activated charcoal. The MS salts have been employed for culture of several more species such as Jatropha curcas (Misra et al., 2010), Punica granatum (Kanwar et al., 2010), Eucalyptus tereticornis (Aggarwal et al., 2010), Pueraria tuberosa (Rathore & Shekhawat, 2009), Pistacia vara (Onay, 2000), Swertia chirata (Wawrosch et al., 1999), etc. In Pentanema indicum, Iiyakkannu et al., 2007 reported maximum callus proliferation on MS medium. MS medium used as basal medium, in Paulownia tomentosa (Rout et al., 2001), Ziziphus spina-christi (Sudhersan et al., 2003), Capsicum annuum (Sanatombi & Sharma, 2005), Aretmisia peterosa (Pace et al., 2004) etc. The concentration of vitamin in MS medium has been increased for establishment of explants of Eucalyptus grandis (Rao & Venkateswara, 1985) and Eucalypyus tereticornis (Das &
Mitra, 1990). Perez-Tornero (2010) examined the influence of MS and DKW media on proliferation stage during micro propagation of nodal explants from mature trees of *Citrus limon* cultivars. They reported that basal media did not affect any of the variables, but the explants on DKW medium were greener. In *Arnebia euchroma*, thidiazuron induced *in vitro* shoot organogenesis when cultured on LS medium (Jiang et al., 2005). In *Cunila incise*, the highest propagation rate was obtained using MS medium supplemented with 4.4 µmol L⁻¹ of benzyladenine (Agostini & Echeverriegaray, 2006). Vengadesan & Pijut, 2009 reported highest shoot regeneration from cotyledonary node explants of *Quercus rubra* cultured on WPM supplemented with BA and GA₃. Bhatt & Dhar, 2004 obtained the maximum number of shoots (4-5/explant) from explants of *Myrica esculenta* was on Woody Plant Medium (WPM) supplemented with 10µM Kinetin and 0.1µM nNaphthalene acetic acid (NAA).

2.1.5 Plant Growth Regulators:

Four broad classes of growth regulators are important in plant tissue culture; the auxins, cytokinins, gibberellins, and abscisic acid. Skoog & Miller were the first to report that the concentration of auxin to cytokinin determined the type and extent of organogenesis in plant cell cultures. Both an auxin and cytokinin are usually added to culture media in order to obtain morphogenesis, although the ratio of hormones required for root and shoot induction is not universally the same. Considerable variability exists among genera, species, and even cultivars in the type and amount of auxin and cytokinin required for induction of morphogenesis. Cytokinins plant hormones promoting cell division and differentiation (Mok & Mok, 2001). The cytokinins include natural and synthetic compounds, adenine derivatives and phenyl urea derivatives. Adenine derivatives are represented by Kinetin, Zeatin and 6-Benzylaminopurine. Phenylurea derivatives include Diphenyl urea or Thidiazuron. The cytokinin production is localized in root tips, shoot meristems, and seeds (Letham, 1994). The single most important medium additives are the cytokinins (Harry & Thorpe, 1994). Most species require exogenous cytokinins to support the growth and development of shoot on establishment medium. Cotyledonary explants of *Brassica oleracea* subsp. *italica* (broccoli) cv. Green Marvel (Ravanfer et. al., 2011) were cultured on MS medium containing different combinations of the growth regulators 6- benzylaminopurine (BAP) and α-naphthalene acetic acid (NAA) for shoot regeneration. Maximum number of shoot was produces per explant with this
medium. Purkayastha et al., 2008 tested various cytokinins (BAP, Kinetin, Thidiazuron and 2-Isopentyl adenine) in MS medium for in vitro multiplication and plant regeneration from nodal explants of Andrographis paniculata. They obtained optimum response at 10 µM of BAP, which induced an average of 34 shoot in 94% of cultures within 4 wk. Enhancement in the induction of shoots by the synergy of BAP and auxins has been documented in Stevia rebaudiana (Sivaram & Mukundan, 2003), Aloe vera (Liao et al., 2004), Murraya koeningii (Rout, 2005) and Euphorbia nivulia (Martin et al., 2005). Kim et al., 2006 studied the effect of PGRs on in vitro shoot proliferation from the axillary bud explants of Hypericum erectum. In this case, the addition of TDZ to culture medium resulted in the induction of more axillary buds than obtained with other cytokinins. Shyamkumar et al., 2003 used different concentrations of BAP to enhance axillary bud initiation in Terminalia chebula.

Pandey et al., 2006 reported that shoot multiplication from nodal explants of T. arjuna occurred on Murashige & Skoog (MS) medium containing different concentrations of 6-Benzyladenine (BA), Thidiazuron or Kinetin, or BA in combination with a-Naphthaleneacetic acid (NAA). The best shoot multiplication response was obtained from nodal explants grown on modified MS (half-strength major salts and Fe-EDTA) medium containing 4.44 mM BA and 0.53mM NAA.

Perez –Tornero et. al., 2010 reported micro propagation from mature nodal segments of Citrus limon on DKW medium with several combination of BAP and Gibberellin. The highest number shoots were produced on medium containing 2 mg l−1 BAP and 1or 2 mg l−1 Gibberellin. Hussain et. al., (2007) carried out evaluation of four different cytokinins viz. TDZ, 2-iP, Zeatin and Adenine sulphate, as a supplement to MS medium for in vitro culture of cotyledonary nodes from 15 days old seedling of Sterculia urens. They found that the use of TDZ at an optimal concentration of 2.27µM was most effective in inducting bud break (83.0%). Faisal et al., 2006 studied the effect MS medium supplemented with 0.5, 1, 2, 3, 4 and 5µM concentrations of TDZ on in vitro shoot proliferation from nodal explants of Psoralea corylifolia. They observed maximum number of shoots in explants placed on MS medium supplemented with 2 µM TDZ. Wang et al., 2008 used combination of TDZ and NAA for in vitro plant regeneration from seedling-derived explants of Boehmeria nivea. They observed highest regeneration efficiency on MS salts with B5 vitamin basal medium containing 2.27 µM TDZ and 0.054 µM NAA. Kamstaityte & Stznys, 2004 observed higher micropropagation of onion ( Allium cepa L.) on MS with
Kinetin than MS with BAP. In leaf culture of *Rehmannia glutinosa*, Park et al., 2009 obtained the highest number of shoots per explants (2.1) and shoot growth (1.2 cm) on MS medium containing 1 mg l⁻¹ TDZ.

Cytokinins are alone as well as used in combination with auxins for the initiation and maintenance of cultures. In combination with auxins, when used at appropriate concentrations, cytokinins can induce shoot regeneration from a callus in angiosperms. Prolific shoot regeneration via organogenesis was reported from leaf and leaf petiole explants of *Astragalus cariensis* on MS medium with NAA and BA (Erissen et al., 2010). The new leaves of *in vitro* grown shoots of *Cornus canadensis* produced calli and adventitious shoot buds on MS medium with 4.4 µM BAP and 0.54 µM NAA (Feng et al., 2009). Addition of Gibberellic acid (1.45 µM) to the same medium, promoted elongation of adventitious buds. Dubey et. al., 2004 reported *in vitro* plant regeneration from apical buds of *Adina cordifolia*. Explants from 3 to 5 years-old trees showed best shoot elongation (1.72±0.04 cm) on MS medium supplemented with 0.5 mg l⁻¹ IBA and 0.5 mg l⁻¹ NAA. They observed on this medium, 46.66% micro-shoot exhibited rooting along with shoot elongation. Rout et al., 2001 obtained clonal propagation from nodal explants of *Paulownia tomentosa* by manipulating the cytokinin and auxin. They observed bud proliferation and multiple shoots from nodal explants derived from greenhouse grown plants of *Paulownia tomentosa* on Murashige & Skoog (MS) medium supplemented with 2.22- 6.66 µM BA. Inclusion of NAA (0.53-1.34 µM) in the culture medium enhanced the rate of multiplication. The shoot length was attained 3-4 cm on MS medium supplemented with 4.44 µM BA + 0.53 µM NAA + 3% (w/v) sucrose, within 4 weeks of culture. Mansor et al., 2003 developed procedures for micropropagation of *Balanities aegyptiaca* using axillary bud explants obtained from mature trees. Cultures were established in Murashige & Skoog (MS) medium supplemented with 2.5 mg l⁻¹ 6-Benzyaminopurine (BAP) and 0.1 mg l⁻¹ Naphthalene acetic acid (NAA).

### 2.1.6 Genotype & Provenance:

Some time in some species genotype and provenance dependent variation observed during *in vitro* propagation. Variation in *in vitro* response of different genotypes has been reported in some other species such as *Frageria x ananassa* (Landi & Mazzetti, 2005), *Alocasia micholitziana* (Thao et al., 2003), *Brassica napus*...
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(Ghanya et al., 2008), *Primula vulgaris* (Schween & Schwenkel, 2003), *Hagenia abyssinica* (Feyissa et al., 2005), etc. Provenance and subculture-dependent variation during micropropagation have been reported in *Gmelina arborea* (Naik et al., 2003). Glowacka et al., 2010 investigated the effects of genotype, inflorescence developmental stage and induction medium on callus induction and plant regeneration in two *Miscanthus* species. They conducted the experiments on three genotypes of *M. sinensis* and one of *M. x giganteus*. The former was represented by genotypes Ms10, Ms17 and Ms20. *M. x giganteus* was represented by MG1. They found that Ms10, using 1-mm inflorescence explants in the developmental stage A and on the MS medium containing 5 mg l\(^{-1}\) 2,4-D and 0.1 mg l\(^{-1}\) BA, about 19 plants can be produced in 22 weeks from a single explants. Under the same conditions, one MG1 explants yields 4.8 plants. Santa-Maria, 2009 reported genotypic variation in meristem culture of high starch sweetpotato (*Ipomea batatas*). Al-Khalifah et al., 2005 reported genotype-dependent in vitro shoot multiplication and elongation in rose. The cultures of Pristine White cultivars produced the highest number of shoots and the cultures of Oklahoma Red cultivars the lowest.

2.1.7 Phenolic Exudation:

Browning of medium and explants occurs due to oxidation of phenols. This is known as phenolic exudation or leaching. Phenolics are secondary metabolites that modulate plant development (Arnaldos et al., 2001) and protect plants against biotic and abiotic stresses (Kefeli et al., 2003; Conceicao et al., 2006; Fan et al., 2006). The injury caused during the excision of explants induces the cells to leach out phenolic compounds, which readily oxidize to produce quinones and cause discoloration. The oxidation products of phenols can be phytotoxic and cause necrosis and eventually death of explant. This exudation can extensively control by polyvinyl pyrrolidone (PVP), Citric acid and ascorbic acid which binds phenols. There are more compounds which control leaching but these are most commonly used. Rathore et al., 2006 incorporated 100.0 mg l\(^{-1}\) ascorbic acid, 25.0 mg l\(^{-1}\) PVP and 0.02% activated charcoal in the MS medium to prevent leaching during tissue culture of *Pueraria tuberosa*. Before inoculating into culture medium, the explants of *Mitragyna parvifolia* (Roy et al., 1988) were suspended in aqueous solution of PVP to check browning. They observed that citric acid and ascorbic acid prevented oxidation of phenols. Abdelwahd et al., 2008 observed leaching of phenolics from the explants of
most genotypes of *Vicia feba* to the culture medium that caused browning, and eventually killed the explants. They pre-treated faba bean seeds with polyvinylpyrrolidone (PVP) and then cultured different types of explants on tissue culture media supplemented with activated charcoal and antioxidants ascorbic acid, cysteine and silver nitrate. They found that the overnight soaked seed with PVP solution for 1 h, followed by culturing in MS medium with sucrose, agar, BA and 2 TDZ, supplemented with ascorbic acid or activated charcoal, greatly reduced lethal browning in explants and improved shoot regeneration. Several workers have suggested solutions to minimize the lethal browning or blackening of explants caused by phenolic compounds in plant tissue culture. Sahini & Gupta, 2002 subcultured stem nodes from in vitro grown plants of *A. catechu* on MS medium adjuvanted with N6-benzyladenine and augmented with 1.5 g l$^{-1}$ PVP to control browning. Explants of *Bambusa balcooa* were treated with different pretreating solutions to increase regeneration frequency that probably hindered due to high phenol content (Das & Pal, 2005). Quraishi et al., 2004 established cultures of *Azadirachta indica* from crown and basal explants on MS medium supplemented with 12.5μM PVP-40, which controlled leaching of phenol growth inhibitors. They also reported that phenolic leaching was not observed in juvenile explants.

### 2.2.1 Proliferation & Elongation of Shoot:

Micropropagation through apical and axillary shoot proliferation is the most common technique for commercial mass production. The ability to rapidly multiply shoot cultures *in vitro* is necessary for establishment of economically feasible micropropagation systems (Harry & Thorpe, 1994). Fast multiplication rates reduced the cost of micropropagated plants (Sommer & Wetzstein, 1984). A shoot tip and an axillary bud having preformed meristems usually develop axillary shoots on a suitable cytokinin concentration with or without auxin. The main factors that contribute to high proliferation rate are genotype, basal medium, plant growth regulator and additives. Rashid et al., 2009 reported multiple shoot induction from shoot tip and nodal segments of *Scoparia dulcis* that were cultured on MS medium supplemented with different concentrations of cytokinins. They found highest number of shoots in nodal segments cultured on MS medium supplemented with 1.0 mg l$^{-1}$ BAP for 8 weeks, and Bodihar et al., 2008 observed highest number of shoot obtain with medium containing 1 mg l$^{-1}$ BAP and 0.25 mg l$^{-1}$ IAA, from nodal explants *Ruta graveolens*. 

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Comparison between BA and TDZ for axillary bud proliferation in *Euphorbia pulchurrima*, revealed maximum response on BA-containing media, whereas TDZ caused shoot hyperhydricity (Pickens et al., 2005). A comparison of BA and Kn on multiple shoot induction in mulberry (*Morus*) showed that BA was superior to Kn (Yadav et al. 1990; Patnaik and Chand 1997). Shende & Rai, 2005 reported multiple shoot formation and plant regeneration in *Buchanania lanzan* (Spreng.) on MS medium supplemented with BAP and NAA. They found that 22.2 µM of BAP and 5.37 µM of NAA promoted formation of the maximum number of shoots. Faisal et al., 2006 developed an efficient plant regeneration system for *Mucuna pruriens* L. using cotyledonary node explants. They observed that maximum shoot proliferation was in 5 µM BAP and 0.5 µM NAA in half strength MS medium at pH 5.8. They also observed that cotyledonary node explants from 7 day old aseptic seedlings induced maximum shoots in MS medium with 5 µM BAP. For rooting, they used 2.0 µM IBA with half-strength MS medium.

A lot of research work has been done from nodal culture for shoot proliferation of several plants (Sanjaya et al., 2005; Siddique et al., 2006). Das & Pal, 2005 produced genetically uniform regenerants of *Bambusa balcooa* using axillary meristem. Liquid MS medium supplemented with 2 mg l\(^{-1}\) BAP, 1 mg l\(^{-1}\) Kn and 8 % coconut water induced axillary bud-break and multiple shoot buds. Successful shoot regeneration and proliferation occurred in liquid MS medium with 3 mg l\(^{-1}\) IBA. For rooting, they tried half strength liquid MS media supplemented with 0.2 mg l\(^{-1}\) IBA. Park et al., 2009 had observed *in vitro* plant regeneration in *Rehmannia glutinosa* from leaf cultures on MS medium containing different concentrations of TDZ. In addition of 1.0 mg l\(^{-1}\) TDZ there was optimal development and growth of shoots. Ogunsola & Ilori, 2008 reported the *in vitro* propagation of embryo and nodal explants using different levels and combinations of auxins and cytokinins in MS medium in *Synsepalum dulcificum* Daniel. Lateral buds proliferation was induced on the germinated embryo with 0.6 - 3.0 mg l\(^{-1}\) BAP + 0.1 - 0.2 mg l\(^{-1}\) NAA in which 3.0 mg l\(^{-1}\) BAP + 0.1 mg l\(^{-1}\) NAA produced highest number of buds.

Iyyakkannu et. al., 2007 reported micropropagation and *in vitro* flowering in *Pentanema indicum* Ling. Maximum callus proliferation was obtained on MS medium supplemented with 2.0 mg l\(^{-1}\) BAP and 1.0 mg l\(^{-1}\) IBA. They achieved best shoot regeneration (19/1.0) in five weeks when callus was cultured on MS medium amended with 4.0 mg l\(^{-1}\) BAP and 1.0 mg l\(^{-1}\) IAA. Hussian et al., 2007 reported shoot
regeneration in *Sterculia urens* Roxb. through cotyledonary node. A proliferating shoot culture was established by repeatedly sub-culturing the original cotyledonary node on shoot multiplication medium (0.45 μM Thidiazuron), after the second harvest of newly formed shoots. Yusuf et al., 2005 reported clonal propagation of *Anogeissus sericea*; multiplication of shoots was done by subculturing shoot, which had two or three nodes after removing their shoot apices, onto MS medium containing 0.56 μM IAA, 6.6 μM BA, 50 mg l\(^{-1}\) IAA, 25 mg l\(^{-1}\) adenine sulphate, 25 mg l\(^{-1}\) arginine and 25 mg l\(^{-1}\) casein hydrolysate. High frequency rooting (97%) was obtained by pulse treating the isolated shoots with 98.0 μM indole-3-butyric acid (IBA) for 6 hours in half strength liquid MS medium then transferring these shoots onto half – strength hormone free semisolid MS medium. Mansor et al., 2003 reported *in vitro* multiplication of forest tree, *Balanites aegyptiaca*, and shoot multiplication required 2.5 mg l\(^{-1}\) BAP, shoot length was significantly affected by the presence of BAP or 6-furfurylaminopurine (Kn.). Rapid clonal multiplication / propagation from mature explants of tree legumes were achieved in *Leucaena leucocephala* (Dhawan & Bhojwani, 1985) etc. Tissue culture technique based on the totipotent nature of cells play a key role in large-scale multiplication of medicinally important plant species (Fracaro & Echeverrigaray, 2001: Liu et al., 2003; Joshi & Dhar, 2003; Faisal et al., 2005; Ahmad & Anis, 2007).

2.2.2 Effect of Subculture on *in vitro* Shoot Regeneration:

After a period of time, it becomes necessary, chiefly due to nutrient depletion and medium drying, to transfer organs and tissues to fresh media. This is particularly true of tissue and cell cultures where a portion of tissue is used to inoculate new culture tubes or flasks; this is known s subculturing. Plant cell and tissue cultures may be maintained infinitely by serial sub culturing. By repeated subculture there can be change in the physiological state and it gradually rejuvenate the shoot, which in turn promotes better rooting. Yusuf et. al., 2010 observed that the numbers of multiple shoots was low during initial subculture but increased after third subculture and were slightly decreased after fourth subculture in *Boesenbergia rotunda* (L.) (a valuable medicinal plant). In *Balanites aegyptiaca*, the highest number of shoots and shoot length up to fifth subculture cycle, when regenerated shoots on MS medium supplemented with optimal concentration of TDZ (5.0 μM) were transferred to
hormone free MS basal medium was reported by Siddique & Anis, 2009. Pierik (1990) studied increase in the percentage of rooted shoots from adult plant with repeated subcultures. The enhancement in shoot multiplication by subsequent cultures is reported in some species are Sterculia urens Hussain et al., 2008; Cardiospermum halicacabum Jahan & Anis 2009; Ocimum basilicum (Siddique & Anis, 2008); Cassia augustifolia (Siddique & Anis, 2007) and Capsicum annuum L. (Siddique & Anis, 2006).

2.3.1 Callus Induction:

Plant callus is a mass of undifferentiated cells derived from plant tissue (explants) for use in biological research and biotechnology. In plant biology, callus cells are those cells that cover a plant wound. Callus is a cluster of undifferentiated, proliferating cells. The callus culture initiates from explants possessing parenchymatous cells capable of renewed cell division. The main factors that influence callus formation are explants type, culture medium and growth regulators. By Kaur, et. al., 2011 an efficient reproducible protocol has been developed for in vitro propagation of endangered medicinal plant (Tylophora indica). They observed that the concentration of plant growth regulators and explant types exhibited discrete roles in the efficiency of plant regeneration. They have also reported that after callus formation it was subcultured on MS medium supplemented with 8.8 μM BAP developed adventitious shoots, however, initially, few shoots were formed but the number increased further on subsequent subculturing. In Melia azedarach Sharry & Teixeira, 2006 optimized a protocol for plant formation from cotyledon explants via organogenesis and somatic embryogenesis. They observed embryogenic callus induction occurred on full strength MS medium containing 3 mgl⁻¹ NAA and 1 mgl⁻¹ BAP with 3 % sucrose with or without 5 mgl⁻¹ Giberrellic acid. Sliwinska et al., 2008 reported efficient plant regeneration through somatic embryogenesis from leaf explants of a 2-yr-old plant of Polyscias filicifolia. They observed embryogenic callus (type I callus) induction on Murashige & Skoog (MS) basal medium supplemented with 0.5 mgl⁻¹ 2,4-Dichlorophenoxyacetic acid (2,4-D) and 1.0 mgl⁻¹ Benzylaminopurine and type II callus on MS medium with 2.0 mgl⁻¹ 2,4-D and 0.01 mg l⁻¹ Kinetin.
2.3.2. Shoot Induction From Callus:

Regeneration of plants by micropropagation of in vitro cultures can be achieved from organ primordia existing in shoot tips and axillary bud explants. Alternatively, plants can be regenerated from unorganized callus tissues derived from different explants by dedifferentiation induced by exogenous growth regulators. Plant regeneration from calli is possible by de novo organogenesis or somatic embryogenesis. Callus cultures also facilitate the amplification of limiting plant material. In addition, plant regeneration from calli permits the isolation of rare somaclonal variants which result either from an existing genetic variability in somatic cells or from the induction of mutations, chromosome aberrations, and epigenetic changes by the in vitro applied environmental stimuli, including growth factors added to the cultured cells. Yusuf et. al., 2011 developed a protocol for high frequency shoot organogenesis and plant establishment from shoot base derived callus was developed for Boesenbergia rotunda (L.). The cultures produced callus in MS medium supplemented with various concentrations of Dicamba and 2,4-D. Multiple shoot induction was achieved from the surface of the callus after transferring onto MS medium supplemented with BAP or Kinetin (shoot induction medium). Dibax et. al.,2011 reported 80% shoot regeneration from embryogenic masses of sugarcane cv. RB931003 and RB98710. Dang et. al.,2011 develop methodologies for the successful in vitro mass propagation of Ilex khasiana a rare and critically endangered holly endemic to the Khasi Hills of Meghalaya, India. They obtained callus from seedling-derived leaf discs cultured on MS medium supplemented with 2,4-Dichlorophenoxyacetic acid and 6-Benzyladenine. Approximately 12 adventitious shoots per callus were regenerated from 83.33 % of the calli after transfer to MS medium supplemented with 6.63 µM 6-Benzyladenine. Karami & Piri,2009 achieved plant regeneration through organogenesis from callus cultures derived from leaf explants of Elaeagnus angustifolia. Dennis & Surabhi,2009 reported multiple shoot induction and callus regeneration in Sarcostemma brevistigma, a rare medicinal plant. An efficient micropropagation protocol was developed for medicinal plant Ipomoea obscura (L.) by in-vitro culture of nodal part of mature plant. The MS media supplemented with 0.8 mgL⁻¹ NAA with 0.8 mgL⁻¹ Kinetin induced three shoots per node in an average and was best for axillary bud proliferation (Mungole,2009). Regeneration of plants from cultured cells occurs via differentiation of shoot- root or
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somatic embryos. Chen, 2006 reported plant regeneration via callus culture from leaf, stem and root explants of *Paphiopedilum* orchids. Leaf, stem and root segments from one-year-old *in vitro* grown seedlings were used to induced callus on MS medium with 2, 4- D and TDZ. Multiple shoot induction from the callus occurred in medicinal crop *Smallanthus sonchifolius* (Jin & Jing, 2006), *Zantedeschia* hybrids (Duquenne et al., 2006), *Cucumis melo* hypocotyl explants (Kathal, 2006), *Furcraea giantea* (Patel et al., 2006) and *Jatropha curcas* cotyledonary and leaf explants (Sharma & Agarwal, 2006). Multiple shoot induction also occurred in *Holestemma annulare* (Sudha et al., 1998), *Crocus sativus* from terminal buds of rest corms (Sheibani et al., 2006), *Dendrobium sonia* from protocorm like bodies of meristem tips (Tee et al., 2006), *Colocasia esculenta* from shoot parts adjacent to corm and shoot meristem (Verma & Cho, 2006). Chand & Sahrawat, 2002 developed an efficient plant regeneration protocol from root explants of *Psoralea corylifolia*, an endangered medicinally important herbaceous plant species belonging to the family *Fabaceae*. Prakash et al., 2002 developed a procedure for plant regeneration of *Hybanthus enneaspermus*, a rare ethnobotanical herb from the Deccan Peninsula in India, through seed-derived callus. Seeds showed a high induction frequency of callus on MS medium containing NAA & BA within 4 weeks of incubation. Shoot differentiation resulted when callus was transferred to MS medium supplemented with BA and NAA multiplied.

2.4.1 Rooting:

Rooting is the key step in micropropagation. Adventitious root formation plays very important role in *in vitro* propagation. An efficient rooting treatment yields a high percentage of rooted shoots and a high-quality root system. The later involves number of roots per shoot, length of roots and absence of callus formation, and determines the performance after planting in soil (Mohammed & Vidaver, 1990; Ven Telgen et al., 1992). Micro-cuttings may be rooted in two ways viz. *ex vitro* rooting and *in vitro* rooting (George, 1996). In *ex vitro* rooting, after a dip in a concentrated auxin solution or in talc-powder with auxin, the microshoots are directly planted *ex vitro*. Usually, micro shoots are rooted *in vitro* on culture medium supplemented with auxin. The major factors that influence rooting of microshoots *in vitro* include age of the explants source, auxin, medium and activated charcoal. Adventitious root formation on a micro cutting is a crucial step in commercial micropropagation. The type of root system formed depends on the physical
characteristics of the rooting environment as well as the species and quality of the micro cutting (George & Sherrington, 1984). Survival of rooted micro cuttings requires that the roots support the plant while new leaves and stems are produced during acclimatization (Nemeth, 1986).

The main factors that influence rooting in micro shoot are - basal medium, concentration of auxin etc.

2.4.1.1 Concentration of Basal Medium:

In some cases roots are unable to initiate in high salt concentration media (Hu & Wang, 1983). When the salt concentrations in the medium get low to one-half, one-third or one-fourth of the strength, rooting becomes abundant. Isikalan et al., 2008 obtained rooting in microshoots of Amygdalus communis cultured on half-MS medium supplemented with (8 mg l\(^{-1}\)) IAA. The tissue culture plants acclimatized in a growth room were successfully transferred to the field. A dilute basal medium is generally used for rooting of microshoots. MS medium with half-strength of macronutrient has been used for rooting the microshoot of Balanites aegyptiaca (Siddique & Anis, 2009), Mitragyna parvifolia (Roy et al., 1988), Dalbergia latifolia (Rai & Chandra, 1989). Hussain et al., 2008 studied rooting in Crotalaria verrucosa. Roots formed on excised shoots when these were cultured on quarter strength MS medium containing IAA. MS medium with 1/3 strength of macronutrients has been used for rooting microshoots of Cardiospermum halicacabum (Jahan & Anis, 2009: Thomas & Maseena, 2006). Hussain et al., 2008 transferred in vitro shoots of Sterculia urens to full strength, half strength and quarter strength MS medium, supplemented individually with IBA, IAA or NAA. Mitra & Pal, 2007 obtained 90% rooting from regenerated shoots of Stevia rebaudiana on half strength MS semisolid medium supplemented with 1.0 mg l\(^{-1}\) IAA within 4 weeks.

Purkayastha et al., 2008 obtained rooting in 94% micro shoots of Andrographis paniculata cultured on MS medium supplemented with 2.5 µM IBA within a week. These plantlets were successfully transferred to soil after hardening with 92% survival rate. Half MS medium has been found suitable for rooting in many species like Cleistanthus collinus (Quraishi et al., 1996; Quraishi & Mishra, 1998), Artocarpus heterophyllus (Amin & Jaiswal, 1993), Withenia somnifera (Wadegaonkar et al., 2006) and Rhodiola fastigiata (Liu et al., 2006). In Acer grandidentatum Clare et al.
, 2007 transferred the shoots to DKW media with IAA for root induction. Thengane et. al., 2006 obtained 52% rooting on WPM medium supplemented with IBA (2.46 – 24.6μM) alone or in combination with BAP (2.22μM) and 77% of plants successfully survived in *Calophyllum inophyllum*. More than 60% *in vitro* shoots of *Feronia limonia* produced roots when cultured on one-quarter strength MS medium containing IBA (Vyas et al., 2005). Hossain et al., 2003 used MS medium at half strength with IBA, IAA, or NAA for root induction in *Zyziphus jujuba*. They found that IBA was more effective in root production compared to others. In *Zehneria scabra* Anand & Jeyachandran, 2004 found MS medium for rooting on in various subculture.

### 2.4.1.2 Type & Concentration of Auxin:

Mainly three type of auxins are more frequently used i.e IAA, IBA or NAA. These are the auxins of choice to achieve rooting in shoots. For rooting of conventional cuttings, IBA is usually applied (Hartmann et al., 1990). IBA is a most commonly used auxin for *in vitro* and *ex vitro* rooting of micro-cuttings (George, 1996). Generally an auxin is added in nutrient for inducing root in microshoot of hardwoods. Indole-3-butyric (IBA) was found more effective in root production compared to others (Hossain et al., 2003). Agrawal & Sardar, 2006 studied rooting in excised shoots of *Cassia angustifolia*. They transferred shoots to a rooting medium containing IAA, IBA or NAA. Nearly 95% shoots developed roots on half strength MS medium supplemented with 10 μM IBA. Ravanfar et al., 2009 reported 100% rooting in shoots of *Brassica oleracea* placed on MS medium with 0.2 mg l⁻¹ IBA. Ramesh et al., 2002 demonstrated the superiority of IBA over the other auxin such as indole-3-acetic acid and NAA in root induction in *Terminalia arjuna*. Thomas & Philip, 2005 When transferred multiple shoots of *Tylophora indica* to the half MS medium supplemented with IBA, NAA or IAA, the optimum rooting response was observed in the presence of IBA. Rashid et al., 2009 revealed that highest number of roots per shoot in *Scoparia dulcis* in half MS medium having 0.5 mg l⁻¹ NAA followed by 1.0 mg l⁻¹ IBA; on the other hand, the lowest number of roots was observed in the full MS medium supplemented with 1.0 mg l⁻¹ NAA. Hussain et al., 2007 reported IBA at 9.80 M was most effective when augmented with quarter strength MS medium is used in *Sterculia urens*. In *Azadirachta indica* (Shahin-uzzaman et al., 2008), *Excoecaria agallocha* (Rao et al., 1998), *Ficus carica* (Kumar et al., 1998), *Balanites aegyptica* (Mansor et al., 2003), *Bambusa vulgaris* (Aliou et al.,
2.4.1.3 Effect of Activated Charcoal:

Activated charcoal might stimulate rooting by absorbing growth regulators and/or inhibitors. Cheruvathur et al., 2010 reported highest frequency of rooting (96%) and mean number of roots per shoot (3.3) in microshoots of *Malaxis acuminata* on MS medium supplemented with 4.0 mg l\(^{-1}\) IBA and 1.5 mg l\(^{-1}\) activated charcoal. Reddy et al., 2001 reported in *Decalepis hamiltonii*, influence of PG and activated charcoal on rooting of *in vitro* derived shoots. They observed when basal region of shoot explants were dipped in IBA solution for 30 min and inoculated on MS basal medium with 0.25% activated charcoal, gave 100% rooting response. Nath et al., 2005 reported in *Adhatoda vasica*, rooting of elongated shoots was assessed by transferring them to full strength MS basal medium containing activated charcoal and 2-3 mg IBA. Activated charcoal when added to the culture medium was found to have a remarkable positive influence on the rooting efficiency (Pati et al., 2006). Reddy et al., 2001, had optimized and standardized an efficient method for rooting of *in vitro* derived micro-shoots of *Decalepis hamiltonii* using phloroglucinol (PG), activated charcoal and CoCl\(_2\).

2.5.1 Hardening or Acclimatization:

Acclimatization phase is critical, because the plantlets undergo a great change in the form of nutrition and in environmental conditions. During this process plants have to adapt to the new environmental conditions such as high light intensity, lower relative humidity, fluctuation of temperatures and microbial attack (Preece & Sutter, 1991). *In vitro* raised plants develop under perfect aseptic condition and high relative humidity (50-70%). When these plantlets are transferred to the field, they sometime die. To overcome this problem; special pretreatment is necessary to harden plantlets. A variety of potting mixture such as peat, perlite, polystyrene beads, vermiculite, fine bark, coarse sand or their mixture in different proportions are used for transplantation. Acclimatization of micropropagated plants on a large scale requires a polyethylene house or glasshouse where misting or fogging system maintains a gradation of high to
low humidity. Amin et al., 2003 transferred Paederia foetida plantlets to plastic pots maintained within a polythene tent for two weeks and then transferred the acclimatized plants to open environment where 80% of the plantlets survived. Ravanfar et al., 2009 successfully acclimatized rooted plants of Brassica oleracea in potting medium containing peat moss, perlite, and vermiculite (3:1:1).

Shukla et al., 2009 transferred the rooted plantlets of Stereospermum personatum to net pots containing mixture of sand: soil: farmyard manure (1:1:1) or coco-peat and placed in green house for primary hardening. After 30 days, survival percentage of tissue culture plants was 83.3 on coco-peat and 50.0 on mixture of sand:soil: farmyard manure. Shukla et al., 2007 acclimatized tissue culture plantlets of Curcuma angustifolia on net pots containing coco-peat substrate and obtained healthy plants. Davood et al., 2008 found the rooted plants of Aloe vera were gradually acclimatized in plastic pots containing a mixture of cocopeat and perlite (1:1) covered with transparent plastic. About 95% of the transplanted plantlets survived. Park et al., 2009 found rooted plants of Rehmannia glutinosa were hardened and transferred to soil with a 73% survival rate. Yusuf et al., 2005 reported that the plantlets were hardened and acclimatized by transferring to autoclaved soilrite moistened with one fourth strength liquid MS mineral salts in glass bottles and then to soil mixture containing sandy soil, black soil and pond soil (1:1:1 v/v) in Anogeissus sericea. To acclimatize the plantlets of Artemisia absinthium, Zia et al., 2007 transferred in vitro rooted plants to pots filled with soil and peat moss (3:1) under high humid condition until maturation of leaves and then transferred to green house.

2.6. Molecular Markers:

Molecular markers have been successfully used to study the source of introduction and variability due to new environment. PCR – based random amplified polymorphic DNA (RAPD) has been widely used to survey genetic structure of populations. Among various molecular markers, the RAPD techniques is simple, rapid and requires only a few nano grams of DNA, has no requirement of prior information of the DNA sequence and has feasibility of automation with higher frequency of polymorphism, which makes it suitable for routine application for the analysis of genetic diversity (Young Um et al., 2001). A variety of DNA markers are available that facilitate assessment of genetic variability in plants. The most common
markers include RFLP (Restriction Fragment Length Polymorphism) and numerous genetic marker assays based on PCR (Polymerase Chain Reaction) such as RAPD (Random Amplified Polymorphic DNA), SSR (Simple Sequence Repeats) and AFLP (Amplified Fragment Length Polymorphism) (Karp et al., 1997).

DNA markers are useful tools for assessment of intra-specific and inter-specific genetic variation, DNA finger printing of clones, authentication of clones, detection of contaminants or substitutes within a clone, detection of somaclonal variation among in vitro regenerated plants, determination of phylogenetic relationship and for plant breeding experiments. RAPD technique has been used extensively used in plants and animals for various purposes. The modified version of this technique utilizes cDNA-RAPD, which amplifies the cDNA instead of genomic DNA. This cDNA modification has also been used to isolate novel genes and perform other genetic studies in plants (Chen et al., 2005, Xie et al., 2002). RAPD markers have also been used in the identification of dwarf genome specific markers in rubber (Venkatachalam et al., 2004). Martins et al.,2003 used RAPD and ISSR markers to analyze the genetic diversity of Prunus dulcis cultivars and their relationship to foreign cultivars. They selected six RAPD and five ISSR primers for their reproducibility and high polymorphism. They found that out of 124 PCR fragments, 120 were polymorphic. All the plants could be discriminated and constitute a very heterozygous group. Wang,2002 reported ISSR markers and their application in different plant genetics. He found that ISSR technique provides quick, reliable and highly informative system for DNA fingerprinting. Verma et al.,2009 used RAPD markers to assess genetic variation in different populations of Trichodesma indicum. Melo et al.,2009 analyzed and quantified genetic variability in four populations of arnica (Lycnophora ericoides) using RAPD markers. They found 35.7% variations among populations and 64.3% variation within populations. Chen et al., 2005 analyzed genetic differences in 19 cultivars selected from somaclonal variants of Syngonium podophyllum along with their parents as well as seven additional Syngonium species and six other aroid using AFLP markers. Various species of genus Trigonella herb are important from medical and culinary aspect. Dangi et al.,2004 assessed genetic diversity in seventeen accessions of Trigonella foenum-graecum and nine accessions of Trigonella caerulea representing various countries using ISSR and RAPD markers. They calculated genetic diversity parameters for ISSR, RAPD and ISSR + RAPD approaches in both the species. The UPGMA analysis revealed that the
distribution of plants from different geographical regions occurred in different groups in both the species. Moon et al., 2010 developed RAPD-derived SCAR (sequence characterized amplification region) markers and multiplex-PCR for rapid molecular authentication of three medicinal plant species Cynachum wilfordii, Cynachum auricalatum and Polygonum multiflorum (Fallopia multiflorum). Similarly, Feng et al., 2010 designed a pair of specific primers from the polymorphic internal transcribed spacer (ITS) regions for molecular authentication of the traditional Chinese medicinal plant Angelica sinensis. They tested eight commercial crude materials and found that four samples were contaminated with regional substitutes. Ramos, et. al., 2011 used microsatellite markers to characterize the effect of first-generation backcrosses of three papaya progeny, by monitoring the level of homozygosity and the parental genomic ratio. They found that linking conventional procedures and molecular markers contributed to an increase in the efficiency of the breeding program in papaya plants (Carica papaya L.).

2.6.1 DNA Extraction:

Preparation of the target DNA template is the first step of RAPD fingerprinting. Isolation and purification of DNA especially from medicinal and aromatic plants is often difficult due to degradation of DNA by endonucleases, co-isolation of highly viscous polysaccharides, inhibitor compounds like polyphenols and other secondary metabolites that directly or indirectly interfere with the enzymatic reactions (Couch & Fritz, 1990).

Initially, minimal DNA template preparation should be necessary for RAPDs since, theoretically, PCR may amplify a single DNA molecule. However, the resulting DNA templates are not very pure and may not be stable for long periods of time. Recently, DNA purity has been implicated as one of the most important factors in RAPD reproducibility (McClelend & Welsh, 1994). He suggest that researchers use only high quality templates to assure reproducible RAPDs. The assay they suggest consists of replicate RAPD PCRs in which the DNA concentration is titrated over two orders of magnitude. If the DNA is of adequate quality the replicates should yield identical RAPD fingerprints.

Number of methods for plant-DNA isolation had been reported which yielding DNA for other enzymatic reaction such as RAPD and RFLP analysis. Generally, these
methods began with grinding of plant tissues in liquid nitrogen followed by incubation in buffers containing detergents such as sodium dodecyl sulphate (SDS) (Dellaporta et al., 1983; Varadarajan & Prakash, 1991), hexadecytrimethylammonium bromide (CTAB) (Doyle & Doyle, 1990; Murray & Thompson, 1980), or a combination of CTAB and SDS (Dellaporta et al., 1983).

Pervaiz, et. al., 2011 standardized a simple and modified method for DNA extraction from the tissue of seeds and leaf of three cereal varieties (Rice, wheat and maize). HwangBo et al.,2010 developed rapid and simple method for DNA extraction from plant and algal species suitable for PCR amplification using a chelating resin Chelex 100. Krizman et. al.,2006 reported a robust CTAB-activated charcoal protocol for plant DNA extraction. Sharma et.al.,2009 reported a simple and efficient method for extraction of genomic DNA from tropical tuber crops. Vural & Dageri,2009 compared four different DNA extraction methods namely the CTAB, Plant Genomic DNA Purification Kit, EZ1 nucleic acid isolation method and DNA extraction with phenol purification and liquid nitrogen method for isolation of DNA for nine aromatic and medicinal plant species growing in Turkey. Bashalkhanov & Rajora,2008 reported a high-throughput DNA extraction system suitable for conifers. Texeira da Silva,2005 analyzed effectiveness 16 DNA extraction methods to extract chrysanthemum, tobacco and Spathiphyllum total genomic DNA.

Narayanan et al.,2006 used four DNA extraction method namely for genomic DNA isolation in Tectona grandis. They observed that CTAB and SDS based extraction procedures extracted better quantity and purity index of genomic DNA. Buragohain & Konwar,2008 standardized the protocol for genomic DNA from fresh young leaves of medicinal plant Meyna spinosa. They used CTAB method for DNA extraction. Kumar et al.,2003 extracted high molecular weight DNA from dry root tissues of Berberis lycicum by following modified CTAB method. Aras et al.,2003 isolated DNA for RAPD analysis from dry leaves samples of Hesperis brevisscapa, H. kotschyi, H. campiara and H. Pendula by CTAB method. Warude et al.,2006 used CTAB extraction method for DNA isolation from fresh as well as commercial samples of Phyllanthus emblica.

Most of the protocols recommend isolation of DNA from fresh tissues. However, sometimes dry or semi dry samples are used. Cheng et al.,2000 used dried rhizome of Lycium barbarum to extract DNA using modified CTAB procedure (Rogers & Bendich, 1985). Aras et. al.,2003 isolated DNA for RAPD analysis from
dry leaves samples of *Hesperis breviscapa, H. kotschyi, H. campiara* and *H. Pendula* by CTAB method. Kumar et al., 2003 extracted high molecular weight DNA from dry root tissues of *Berberis lycicum* by following modified CTAB method. Jogaite et al., 2005 extracted DNA from fresh leaves of *Paris quadrifolia* using genomic DNA Purification Kit (Fermentas, Lithuania). Deng et al., 2006 extracted DNA from 180 individuals of *Lycoris longituba*, representing three populations namely Langyashan population, Xuyi population and Baohuashan population. Warude et al., 2003 reported a modified method for DNA isolation from fresh leaves of *Emblica officinalis, Terminalia bellerica* and *T. chebula*, which have low pH and high amounts of secondary metabolites in tissue extracts. They found that this method yields good quality, high-molecular weight DNA that is free of contaminants and colored pigments and is suitable for PCR amplification.

### 2.6.2 Random Amplified Polymorphic DNA:

**Taq polymerase** is a thermostable DNA polymerase named after the thermophilic bacterium *Thermus aquaticus* from which it was originally isolated by Thomas D. Brock in 1965. Frequently used in polymerase chain reaction (PCR), a method for greatly amplifying short segments of DNA. Taq polymerase was identified as an enzyme able to withstand the protein-denaturing conditions (high temperature) required during PCR. Therefore it replaced the DNA polymerase from *E. coli* originally used in PCR. Taq DNA Polymerase is highly purified free of contaminating endonucleases, exonucleases and nicking activity. The basic concept was tested for the first time with Klenow polymerase but the real breakthrough came when a thermostable DNA polymerase, *Taq* polymerase (Mullis & Fallona, 1987), was isolated and purified.

The Randomly Amplified Polymorphic DNA (RAPD) technique, described first by Welsh & Mcclelland (1990) and Williams et al. (1990), is a quick and effective method for producing species-specific fingerprints. It is based on a modified PCR method and uses short oligonucleotide primers of arbitrary sequence to amplify anonymous fragments of genomic DNA. It is immediately applicable to the analysis of most organisms because universal sets of primers are used without any need for prior sequence information (Nybom, 2004). Gurudeeban, 2011 has evaluated the genetic variability among three salt marsh plant (*Suaeda sp.*) belongs to the family of Chenopodiaceae using random amplified polymorphic DNA markers. RAPD protocol
was optimized based on the use of lower concentrations of primer (2 μM) and Taq polymerase (2 units), 50 ng of template DNA. The requirement of enzyme may vary with respect to individual target template or primer. Wang el al., 2010 used 1.0, 1.5 and 2.0 units to optimize Taq DNA polymerase concentration for RAPD-PCR of 40ng genomic DNA from 15 Camellia cultivars. The Taq polymerase optimised at 1.5 units in reaction mixture. Das et al.,2009 reported that 1.5unit Taq DNA polymerase was optimum for 50ng genomic DNA of banana cultivars. Wang et al.,2005; Nanda et al.,2004) used 1.0 unit of Taq DNA polymerase for RAPD analysis of Neolitsea sericea and Acacia mangium. Ginwal & Maurya,2010 reported 4ng genomic DNA and 5 unit Taq polymerase for RAPD and ISSR assay of Dalbergia sissoo. Sharma et al.,2008 used 80 ng genomic DNA and 1.0 unit of Taq polymerase for RAPD analysis of Andrographis paniculata. Whereas 0.2 and 0.5 units Taq DNA polymerase was used for RAPD analysis of Phyllanthus amarus (Jain et al., 2003) and Hypericum perforatum (Haluskova & Kosuth, 2003) respectively. Verma et al.,2009 used 20ng DNA and 0.6 unit Taq polymerase for RAPD analysis of Trichodesma indicum. Haluskova & Kosuth,2003 used 0.5unit Taq DNA polymerase for RAPD analysis of Hypericum perforatum, whereas Jain et al.,2003 used 0.2unit Taq DNA polymerase for RAPD analysis of Phyllanthus amarus. This marker system was also used in many different applications involving the detection of DNA sequence (Zhou et al., 2007).

2.6.2.1 Polymerase Chain Reaction Amplification of DNA:

The polymerase chain reaction (PCR) is a relatively simple technique that amplifies a DNA template to produce specific DNA fragments in vitro. Traditional methods of cloning a DNA sequence into a vector and replicating it in a living cell often require days or weeks of work, but amplification of DNA sequences by PCR requires only hours. While most biochemical analyses, including nucleic acid detection with radioisotopes, require the input of significant amounts of biological material, the PCR process requires very little. Thus, PCR can achieve more sensitive detection and higher levels of amplification of specific sequences in less time than previously used methods. These features make the technique extremely useful, not only in basic research, but also in commercial uses, including genetic identity testing, forensics, industrial quality control and in vitro diagnostics.

The PCR process was originally developed to amplify short segments of a longer DNA molecule (Saiki et .,al 1985). A typical amplification reaction includes
target DNA, a thermostable DNA polymerase, two oligonucleotide primers, deoxynucleotide triphosphates (dNTPs), reaction buffer and magnesium. Once assembled, the reaction is placed in a thermal cycler, an instrument that subjects the reaction to a series of different temperatures for set amounts of time. This series of temperature and time adjustments is referred to as one cycle of amplification. Each PCR cycle theoretically doubles the amount of targeted sequence (amplicon) in the reaction. Ten cycles theoretically multiply the amplicon by a factor of about one thousand; 20 cycles, by a factor of more than a million in a matter of hours.

Each cycle of PCR includes steps for template denaturation, primer annealing and primer extension. The initial step denatures the target DNA by heating it to 94°C or higher for 15 seconds to 2 minutes. In the denaturation process, the two intertwined strands of DNA separate from one another, producing the necessary single-stranded DNA template for replication by the thermostable DNA polymerase). Padmalatha & Prasad (2006) optimized PCR protocol for many medicinal plants. They programmed thermocycler for an initial denaturation step of 3 minutes at 94 °C, followed by 30 cycles of 45 s at 94 °C, 1 minute at 37 °C, extension was carried out at 72 °C for 1 minute and final extension at 72 °C for 7 minutes and a hold temperature of 4 °C at the end. For RAPD of Phoenix dactylifera, Ahmed et al., 2009 used 45 cycles of 30 s at 94°C for the denaturation, 1 minute at 37°C for primer hybridization and 2 minutes at 72°C for complementary strands synthesis and programmed final elongation at the end of the last cycle amplification for 10 minutes. In the next step of a cycle, the temperature is reduced to approximately 40–60°C. At this temperature, the oligonucleotide primers can formtable associations (anneal) with the denatured target DNA and serve as primers for the DNA polymerase. This step lasts approximately 15–60 seconds. Finally, the synthesis of new DNA begins as the reaction temperature is raised to the optimum for the DNA polymerase. For most thermostable DNA polymerases, this temperature is in the range of 70–74°C. The extension step lasts approximately 1–2 minutes. The next cycle begins with a return to 94°C for denaturation.

Each step of the cycle should be optimized for each template and primer pair combination. If the temperature during the annealing and extension steps are similar, these two steps can be combined into a single step in which both primer annealing and extension take place. After 20–40 cycles, the amplified product may be analyzed for size, quantity, sequence, etc., or used in further experimental procedures.
2.6.2.2 DNA Concentration:

The amount of template required for successful amplification depends upon the complexity of the DNA sample. Reactions with too little DNA template will have low yields, while reactions with too much DNA template can be plagued by nonspecific amplification. For most species of plants, good results have been achieved using 50 to 100 ng in 50 µl reaction mixtures. About 15ng of genomic DNA is used for *Lablab purpureus* (Rai et al., 2010) and 10-20 ng for *Cynachum wilfordii*, *C. auriculatum* and *Polygonum multiflorum* (Moon et al., 2010). In RAPD analysis of *Cymbopogon* species, 30ng genomic DNA was used (Kumar et al., 2009) and in the amplification reaction of *Sequoia sempervirens*, 30-50ng DNA was used (Toral Ibanez et al., 2009). Padamlatha & Prasad, 2006 optimized RAPD-PCR reaction for *Vitex pubescens*, *Nervilla aragoana*, *Gynema sylvestre*, *Withenia somnifera*, *Origanum majorana*, *Boswellia serrata*, *Saraca asoca* and *Gloriosa superba*. They found that 50ng of template DNA was optimum for PCR reaction, whereas higher concentrations produced smear and lower concentrations showed absence of amplified bands. In *Changium smyrnioides*, Fu et al., 2003 used 40ng of template DNA. In case of *Echinaceae purpurea* 15 µl reaction volume contained about 50 ng of template DNA (Vural & Dageri, 2009). The PCR reaction mixture consisted of 50 ng genomic DNA as template in *Oroxylum indicum* (Jayaram & Prasad, 2008).

2.6.2.3 PCR Buffer:

A general recommendation for PCR is 10 mM Tris-HCL at pH 8.0 (Chengxin et al., 2003). However, use of increased Tris-HCL concentration (Echt et al., 1992), and higher pH (Demeke et al., 1992) have been reported. 1.25 mM dNTPs is used in *Paulownia tomentosa* (Rout et al., 2001). For PCR 10 mM Tris-HCl, 50 mM KCl and 1.0 mM MgCl₂ used in *Scaevola hainanensis* (Kuen- Yih, 2005). Batitini et al., 2009 reported protocol for *Anemopaegma arvense* 3.0 µL buffer 10X; 3.0 µL dNTPs (2.5 mM); 1.8 µL MgCl₂ (25.0 mM); 4.0 µL primer (10 ng/µL); 0.3 µL *Taq* enzyme.

2.6.2.4 Optimization of the PCR:

RAPD analysis needs careful optimization because the amplification is generally less than optimal when a PCR is set up for first time with a new template DNA, new primers, or a new preparation of thermostable DNA polymerase
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(Sambrook & Russell, 2001). There is no one optimum protocol for all species. Some of the difference in protocol may be due to variation in thermal cyclers or the source of Taq- polymerase, but the main difference is the species under study. It is clear that the polymerase chain reaction is composed of a number of interacting components which includes not only the reaction itself, but the sample preparation as well (Linz, 1991). Therefore, a PCR reaction is optimized for individual species. The seven essential components of PCR are a thermostable DNA polymerase, synthetic decanucleotide primers, deoxynucleoside triphosphates, divalent cations, buffer to maintain pH, monovalent cations and template DNA (Sambrook & Russell, 2001). The PCR conditions for RAPD analysis can be optimized by varying the many components, namely, type and concentration of thermostable polymerase, deoxynucleotide triphosphates (dNTPs), Mg$^{2+}$ ions, primer and DNA template concentration, and other reaction factors such as primer annealing, primer extension, denaturation time and temperature. All these factors can influence a PCR reaction, and exacerbated by the fact that not all processes and mechanisms involved in such reactions are as understood fully (Wolff et al., 1993).

2.6.2.5 RAPD Polymorphism:

RAPD is a common molecular approach employed in DNA fingerprint analysis for genotypic differentiation, molecular taxonomy, and other applications (Lin et al., 1996). Arya et. al.,2011 reported Intra-specific Genetic Diversity of Different Accessions of Cassia occidentalis by RAPD Markers. Cesar et al.,2009 assessed genetic integrity of micropropagated plants of Bacopa monnieri by RAPD. They carried out RAPD analysis with five random primers and found monomorphic amplified products that were similar to those of mother plant. Maia et al., 2009 reported genetic diversity in somatic mutants of grape Vitis vinifera based on random amplified polymorphic DNA. The 10 primers used for RAPD fingerprints generated 126 reproducible fragments, of which 63, 68, 76, and 72 were polymorphic. Among the primers, OPP-08 generated the highest number of fragments, whereas OPE-15 was the most efficient for discriminating polymorphic fragments. The distribution of the clones by cluster analysis indicated that there were no differences in RAPD markers between the colored mutant and the original clone. They found high levels of polymorphism within and between the cultivars Italia, Rubi, Benitaka, and Brasil.
(65.1%), contrary to a previous hypothesis that the four clones are genetically uniform. Genetic variability in a medicinal plant *Artemisia judaica* using Random Amplified Polymorphic DNA (RAPD) Markers was reported by Al-Rawashdeh, 2011.

Random amplified polymorphic DNA (RAPD) markers were applied to detect the genetic relationships and diversity among 33 accessions of six medicinal species of *Curcuma* in China, by Zou, et. al., 2011. Pandey et al., 2008 assessment genetic diversity in Indian ash gourd (*Benincasa hispida*) accessions using RAPD markers. He studied thirty-four (09 cultivars and 25 germplasm) genotypes of *Benincasa hispida* to assess the molecular diversity using RAPD markers. Twenty-five RAPD markers produced 163 amplicons, of which 73% were polymorphic indicating a high degree of diversity. The dendrogram revealed that the accessions from northeastern regions of India were different from the accessions of other parts of India, as they clustered together. The clustering indicated that cultivars had a narrower genetic diversity among themselves as compare to germplasm collected from different agro-ecological region. Ponnumswami et al., 2008 reported genetic relationship and diversity in palm in palm (*Borassus flabellifer*) accessions based on RAPD markers. They obtained 57 bands, among them 43 were polymorphic, and size of amplification products ranged from 250 to 3200 bp. UPGMA based cluster diagram showed in all 20 different genotypes grouped in 04 different clusters based on the stature, sex and high yielding types. Lakshmanan et al., 2007 reported complete uniformity among the regenerates and also between the regenerates and field grown mother clone in *Musa spp.*

Naugzemys et al. (2006) reported comparison of DNA polymorphism in seedling of *Pinus sylvestris* L. from different population by RAPD markers. Random amplified polymorphic DNA to examine genetic differences among three populations of *Pinus sylvestris* L. They identified that 63 RAPD loci in 90 plants. All individuals had specific RAPD phenotype. Allele frequency data were used to calculated within-population and within-species genetic diversity. The level of average heterozygosity was highest in Lanbanoras population and the lowest in Telsiai population. The UPGMA dendrogram based on the genetic distance and PCO analysis gave similar results and showed that Druskininkai and Telsiai population were most genetically distant.
Prathepha (2000) detected RAPD variation in a forest tree species *Melientha suavis* Pierre from Thailand. Random amplified DNA marker to investigate the patterns and distribution of genetic variability in natural population of *M. Suavis*. They observed seven 10-mer primer amplified a total of 46 scorable bands, of which 36 (78.3%) were polymorphic. Results from Shannon’s information measures indicated that twenty-eight percent of genetic variations were found within population. AMOVA analysis revealed most genetic variation was found within population comparable to the total genetic variation.

Bhalla, 2007 concluded that RAPD analysis could establish the clonal integrity of tissue cultured generated plants. They could not detect any polymorphism when the banding patterns of stock plants were compared to their in vitro-derived progeny in *Chlorophytum arundinaceum*, RAPD analysis revealed similar banding profile between micro propagated plants and the mother plant (Lattoo et al., 2006). Martins et al., 2004 studied the genetic stability of micropropagated *Prunus dulcis* plantlets using RAPD and ISSR markers. Sixty four RAPD and 10 ISSR primers produced 326 distinct, reproducible, and monomorphic bands across all the regenerates. No variation was also observed among the regenerants of *in vitro* cultured *Bambusa balcooa* (Gillis et al., 2007). Another recent report on somaclonal variation in *Vanilla planifolia* (Reddampalli et al., 2007) reported genetic uniformity among the micropropagated plants within one genotype. No variation was observed among the regenerants using both RAPD and ISSR markers. Ray et al., 2006 studied the genetic stability among micropropagated *Musa* cultivars. Using a total of 21 RAPD and 12 ISSR primers that amplified 5330 RAPD and 2741 ISSR loci, they could detect genetic stability in one cultivar while the plants derived from meristem culture from the other two cultivar were not true to-type. Govarthanan, et. al., 2011 assess molecular variation among *C.amboinicus, C.aromaticus and C.forskohlii*. and determine the level of genetic similarity among them. They performed random amplification of polymorphic DNA (RAPD) analysis on three strains of *Coleus sp*. Random primers were used for the PCR. The RAPD technique was used to find out the extent of genetic diversity in *coleus sp*. And primers OPW (6-10) and OPU were used for amplification. Maximum number of bands were observed *Coleus amboinicus* with primer OPW 6 followed by OPW 7. A sum total of 28 bands were amplified with respect to all the 5 primers (OPW 6-10). By using OPW (6-10) total number of bands amplified were 28, where as there was no amplification in OPU (15-19).
variation among *coleus* *sp* was identified from OPW but not in OPU primers. In *Coleus amboinicus* totally 15 bands were amplified, in *Coleus aromaticus* there was no amplification, *Coleus forskohlii* 13 bands were amplified.

These studies clearly demonstrated that DNA amplification techniques like RAPD could be used to detect genetic diversity in micropropagated plants and different population of plants. RAPD which has been proved to be a potential DNA marker to determine the genetic variability among the regenerates.