Review of literature
Curculigo orchioides is one of the important medicinal herbs for Ayurvedic Materia Medica (Irshad et al, 2006). The leaves, roots and rhizome of Curculigo orchioides are medinally useful (Bhamare, 1998; Jain, 1991). According to Unani system of medicine, root is carminative tonic, aphrodisiac, antipyretic, and used in bronchitis, ophthalmia, indigestion, vomiting, diarrhea, lumbago, gonorrhea, gleet, hydrophobia and joint pain.

It is an important medicinal herb by virtue of anticancerous properties of its rhizome (Prajapati et al, 2003). The anticancerous property was observed against Sarcoma 180 mouse (Dhar et al, 1968). Adaptogenic, anti-inflammatory, antioxidant (Venkumar and Latha, 2002a), hepatoprotective (Venkumar and Latha, 2002b), anticonvulsant, sedative, androgenic and immunopromoting activities for the plant was reported (Xu et al, 1992a; Xu et al, 1992b). Its potential was cited as biopesticide (Ignacimuthu, 1998).

The tuberous root stock of Curculigo orchioides is used as a restorative, rejuvenating and aphrodisiac drug (Porwal and Mehta, 1985; Porwal et al, 1988; Manandhar, 1991; Samanta, 1992). It cures morbid vata and
pitta, improves complexion and is useful debility, deafness, cough, asthma, piles, skin diseases, jaundice, urinary disorders, leucorrhoea and menorrhagia (Nadkarni, 1954; Anonymous, 1963; Moos, 1978; Srivastava, 1989; Banerjee and Pal, 1994; Govil, 1998).

Thin slices of the rhizome without root hairs are employed in drug formulations like Vidaryadighrta, Vidaryadilehya, Marmagulika, Musalyadichurna etc (Porwal and Mehta, 1985; Sivarajan and Balachandran, 1994; Warrier et al, 1994). It delays ageing process and forms ingredient of many health foods and other preparations (Nadkarni, 1954; Nagarajan et al, 1982; Jogleker et al, 1984; Kumar and Prabhakar, 1990; Sharma et al, 1991). Rhizomes have the properties of warming the kidneys, invigorating expelling cold, and eliminating dampness, and are used as the traditional Chinese medicine xianmao to cure impotence, cold sperm, cold pain of the back and knee, and numbness of the limbs (Liu, 2001). It is used with the other drugs for general and sexual weakness, leucorrhoea and menstrual disorders etc. It is the ingredient of many unani formulations viz Majun Mochras used for leucorrhoea, Majun samagah
and *Majun Pumb Dana* used as an aphrodisiac and *Hab-Asgand* prescribed in lumbago, rheumatism and gout (Misra *et al.*, 1984a)

The ethanolic extract of rhizome of *Curculigo orchioides* is reported to have anti-hyperglycemic (Chauhan and Dixit, 2007) and antiasthmatic activity (Pandit *et al.*, 2008).

The methanolic extract of rhizome is reported to have immune-stimulant principles (Lakshmi *et al.*, 2003; Bafna and Mishra, 2005). It has been shown to enhance phagocytic activity of macrophages. The pure glycoside fraction of the methanolic extract has been found to stimulate immune response by acting both on macrophages and the lymphocytes. In addition to this, the aphrodisiac activity of *Curculigo orchioides* is observed in male albino rats. Moreover, anti-oxidant, anti-fungal, anti-microbial, anti-tumor, anti-diabetic and anti-bacterial activity of methanolic extract of this plant was screened (Venukumar and Latha, 2002a; Venukumar and Latha, 2002b; Bafna and Mishra, 2005; Singh and Gupta, 2008). Antifungal activity was screened using agar plate method, and antibacterial activity of the extracts was
determined by disk diffusion method. Antitumor activity was screened against a human breast cancer cell line (Singh and Gupta, 2008).

Furthermore, the estrogenic activity of alcoholic extract of this medicinal herb was studied (Vijayanarayana et al, 2007). Alcoholic extract of rhizome showed a significant increase in percentage vaginal cornification, uterine glycogen content, and proliferative changes in uterine endometrium.

The curculigoside exhibited potent inhibitory activity against matrix metallo-proteinase 1 in cultured human skin fibroblasts. In addition, it increased the level of Bcl-2 protein expression and decreased the level of Bax protein expression (Lee et al, 2009).

1. Phytochemical analysis
Many researchers have carried out phytochemical analysis of this endangered medicinal plant. The rhizome contains curculigoside, a phenolic glycoside characterized as 5-hydroxy-2-O-β-D-glucopyranosyl benzyl 2, 6-dimethoxy benzoate (Oru and Kogyo, 1983; Chen et al, 1989; Mamta et al, 1995; Chen and Ni, 1999). It also contains a flavone glycoside (Dhawan and Saxena, 1958) and saponin
G (Xu et al, 1992b). A method for the determination of curculigoside from the crude medicine by ultrasonic vibration was established (Lu et al, 2002). Curculigoside A and Curculigoside B from rhizome of *Curculigo orchioides* was isolated, separated and purified by HSCCC (Peng et al, 2006). Structure of the Curculigoside was elucidated as 5-hydroxy-2-O-b-D-glucopyranosyl benzyl-3'-hydroxy-2',6'dimethoxy-benzoate by using spectroscopic methods (Kubo et al, 1983). The rhizome yielded a phenolic glycoside (orchioside), characterised as orcinol-3-D-xylopyranosyl-(1,-β-6)-D glucopyranoside and hentriacontanol (Garg et al, 1989).

Three new compounds from rhizome was isolated and identified as N-acetyl-N-hydroxy-2-carbamic acid methyl ester, 3-acetyl-5-carbomethoxy-2H-3,4,5,6-tetrahydro-1,2,3,5,6-oxatetrazine and N, N, N', N'-tetra methyl succinamide (Porwal et al, 1988). An aliphatic compound has been isolated from the rhizomes and characterised as 25-dihydroxy-33-methyl pentatricontan-one (Mehta et al, 1990). A natural triterpene alcohol- curculigol was isolated from the rhizome and characterised as 24-methyl cycloart-7-en-3-beta-20-diol (Misra et

A novel pentacyclic triterpene and 17 cycloartane (saponin A-J) was isolated from the rhizomes of \textit{Curculigo orchioides} and characterised as 31-methyl-3-oxo-20-ursen-28-oic acid (Mehta and Gawarikar, 1991; Xu and Xu, 1992a; Xu and Xu, 1992b; Xu \textit{et al}, 1992a; Xu \textit{et al}, 1992b). Two new aliphatic hydroxy-ketones, isolated from the rhizomes have been characterized as 27-hydroxytriacontan-6-one and 23-hydroxytriacontan-2-one, respectively (Misra \textit{et al}, 1984b).

Root stock of \textit{Curculigo orchioides} was reported to contain glucose, mannose, xylose, glucuronic acid, glycoside, polysaccharides (hemicellulose and other polysaccharides), starch, resin, tannin,
mucilage, fat and calcium oxalate (Rao and Beri, 1951; Thakur et al, 1989).

Fig- 4: Phenolic glycosides isolated from Rhizome of Curculigo orchioides. 1. curculigoside, 2. curculigosides B, and 3.curculigosides C, 4. orcinol glycoside and 5. anacardoside.

Curculigoside: (5-hydroxy-2-O-β-D-glucopyranosylbenzyl-2,6 dimethoxy benzoate ) or (2,6-dimethoxy benzoic acid)

Molecular formula: C$_{22}$H$_{26}$O$_{11}$

Molecular weight: 446
The flavone glycoside from the rootstock has been identified as 5, 7-di-methoxy gluco pyranoside (Yadav et al, 1974; Sharma et al, 1975). The hexane extract contain an alkaloid-lycorine, sterols including-sitosterols and sapogenin identified as yuccagenin (Chopra et al, 1956; Rao et al, 1978; Husain et al, 1992).

A number of fatty acids have been isolated from *C. orchioides* root oil by GLC techniques (Mehta et al, 1980). They were palmitic, oleic, linolenic linoleic, arachidic and behenic acids. Two aliphatic hydroxy ketones, 27-hydroxy tricontan-6-one and 23-hydroxy tricontan-2-one, were isolated from the rhizome of *C. orchioides* (Misra et al, 1984a; Misra et al, 1984b). They further isolated 21-hydroxy tetracontan-20-one and 4-methyl heptadecanoic acid from the root stock.

The root tubers of this plant was analysed by EDX analysis (Agrahari et al, 2010). The EDX analysis showed that root tubers possesses only seven (C, O, Cl, K, Ca, Cu & Zn) essential elements. The root tubers were found deficient in Mg, Al, Si and Fe.
2. Plant tissue culture

Plant tissue culture has found its application in rapid clonal propagation (Murashige and Skoog, 1962), raising disease resistant plants (Kartha and Gamborg, 1978), anther culture for haploid production (Guha and Maheshwari, 1964), somatic embryogenesis for synthetic seed production (Sharp et al, 1982), secondary metabolites of immense value (Constable et al, 1974), somaclonal variation for the recovery of novel genotypes (Larkins and Scowcroft, 1981) protoplast culture and fusion to obtain somatic hybrids (Kumar and Cocking, 1987) etc.

*Curculigo orchioides* is an endangered seasonal herb, growing only during monsoon (Dhar et al, 1968). It is propagated by underground rhizomes or seeds. Rhizomes of this plant are prone to viral and bacterial diseases. The seed set is very poor. Hence the methods for large scale *in vitro* propagation are needed to meet the demand and to conserve the valuable plant. Tissue culture approaches have been vital in the re-establishment of endangered plant species.

The protocol for the micropropagation of *C. orchioides* was first standardized by Augustine and D'souza (1997b). The protocol was later on revised/modified by several researchers. *Curculigo orchioides* can be propagated by direct organogenesis, indirect organogenesis somatic embryogenesis and bulbil formation.

### 2.1. Direct organogenesis

Direct organogenesis was achieved through leaf, rhizome and meristem tip culture (Augustine and D'souza, 1997b; Nagesh *et al*, 2009).

Augustine and D'souza (1997b) established the protocol for the micropropagation of *Curculigo orchioides* from the leaf and rhizome explant. They successfully regenerated plantlets from leaf explants maintained on MS medium. Generated shoots were rooted on MS
medium supplemented with NAA/IAA/IBA. Nodular callus formation from rhizome was reported. The role of polarity in de novo shoot bud initiation from stem disc explants of *Curculigo orchioides* was reported (Nagesh *et al*, 2009). Moreover seed as an explant was utilized for regeneration of *Curculigo orchioides* (Rekha and Reddy, 1998).

### 2.2. Indirect organogenesis

Callus formation in *Curculigo* has been initiated mostly through leaf (Augustine and D’souza 1997b; Prajapati *et al*, 2003; Prajapati *et al*, 2004; Dhenuka *et al*, 1999; Thomas, 2007a), rhizome (Augustine and D’souza, 1997b; Prajapati *et al*, 2004; Thomas, 2007b) and stem disc (Suri *et al*, 1999). Higher level of BA induced callus formation in both leaves and rhizome explants (Augustine and D’souza, 1997b). High frequency of callus formation was obtained from leaf explants rather than rhizome. Different concentration of NAA, BA, 2, 4D and KIN, were tried for better callus formation (Prajapati *et al*, 2004). The non-morphogenic greenish white callus was obtained on MS medium supplemented with NAA (2 mg/l) + BA (1 mg/l) while friable callus was achieved on MS media supplemented with 2, 4D (2 mg/l) from leaf
explants (Prajapati et al, 2004). Through indirect organogenesis maximum 8 shoots were obtained (Prajapati et al, 2004). Pretreatment of leaf explant with TDZ resulted in improved shoot regeneration (Thomas, 2007a). Pretreatment of leaf explant with TDZ (15 µM) for 24 hours significantly promoted the formation of adventitious shoots (16.2 shoots/explant) and 96 % response on MS medium supplemented with TDZ (6 µM).

2.3. Somatic embryogenesis

Somatic embryos were developed from the leaf explant of Curculigo orchioides (Suri et al, 1998; Thomas and Jacob, 2004). The leaf segments transferred to the liquid medium supplemented with BA (2.2 µM), 2,4,5-T (1 µM) and IBA (1 µM) produced somatic embryos (Suri et al, 1998). The combination of BA and IBA inhibited somatic embryogenesis but favored bulbil formation. Lower concentration of nitrogen promoted both bulbil formation and somatic embryogenesis. BA (8 µM) gave optimum response in terms of percent cultures responding (81 %) and the number of embryos (16) per explant (Thomas and Jacob, 2004). Several somatic embryos were emerged
from the adaxial side of the leaf explant after one month of culture (Thomas and Jacob, 2004).

2.4. Bulbil formation

Direct and indirect bulbil formation from leaf explant was studied in *C. orchioides*. (Suri *et al*, 2000; Nema *et al*, 2008; Thomas, 2007a). Morphactin and cytokinin promoted high frequency bulbil formation in shake flask cultures (Nema *et al*, 2008a). B5 medium supplemented with BA, IBA, Adenine and PVP induced direct bulbil formation (Suri *et al*, 2000) while MS medium supplemented with 1.0 mg/l IBA with 0.1 mg/l substituted urea promoted callus formation. This medium supports maximum percent explant response (100 %), bulbil number per explant (28.5) and yield of bulbils (Nema *et al*, 2008). Different cytokinins (KIN, BAP, TDZ), Auxins (IBA, NAA) and Sugars (Sucrose, Mannose, Glucose) were utilized to enhance bulblet production (Thomas, 2007a). The maximum result were obtained with the MS medium supplemented with TDZ (7 µM), IBA (0.5 µM) and mannose (200 mM). On this medium, 97 % of the cultures responded with an average number of 26.8 bulblets per culture.
2.5. Shoot tip culture

*In vitro* propogation of *Curculigo orchioides* through meristem tip culture was studied (Wala and Jasrai, 2003; Fransis et al, 2007; Sharma et al, 2007). Multiple shoots were obtained from the meristem tip culture on MS medium supplemented with 2.21 µM BA (Wala and Jasrai, 2003). Various growth regulators were supplied to the basal medium to improve shoot multiplication through meristem tip culture (Fransis et al, 2007; Sharma et al, 2007). The highest frequency of multiplication was obtained on MS medium supplemented with BA (1.5 mg/l), AD (100 mg/l), IBA (0.25 mg/l). Explants cultured on MS + BA (0.2 mg/l) + spermidine (2.5 mg/l) produced multiple shoots with an average of 7 shoots/expilants (Sharma et al, 2007).

2.6. Arbuscular mycorrhizal (AM) technology

This technology was used for the conservation of *Curculigo orchioides* (Sharma et al, 2008b). Effect of three AM fungal inocula on post transplanting performance of *in vitro* raised *Curculigo orchioides* plantlets was reported. The three AM fungal inocula consisted of two monospecific cultures of *Glomus geosporum* and *Glomus microcarpum*
and one crude consortium of AM fungal spores isolated from rhizosphere soil of *Curculigo orchoides* growing in natural habitat. Plantlets responded significantly different to all three mycorrhizal treatments. Mycorrhization enhanced the survival rate of *Curculigo orchoides* plantlets to 100 %. The inoculated plantlets fared significantly better than the un-inoculated ones in terms of biomass production and number of leaves and roots per plant. The mixed consortium of AM fungi consistently performed better in terms of the number of leaves, number of roots, fresh and dry mass of shoots and roots.

The effect of elicitors (jasmonic acid, salicylic acid and ethephon) on curculigosides production in *in vitro* grown plantlets of *Curculigo orchoides* was studied (Nema *et al*, 2008b). The salicylic acid enhances maximum curculigoside accumulation followed by methyl jasmonate (MJA) and ethephon.

### 3. Field performance of tissue cultured plants

Field performance of tissue cultured plants were studied in number of species (Msogoya *et al*, 2011; Maximova *et al*, 2008; Arce-Montoya *et
al, 2006; Sood et al, 2006; Gustavsson and Stanys, 2000). Agronomic parameters studied in one year tissue cultured sugarcane (Sood et al, 2006). The first generation canes were of exceptional quality as seed cane. The plantlets grew early and within a short time abundant numbers of tillers developed in a synchronous manner. The plant height and of the tissue cultured sugarcane is significantly higher than that of offset raised sugarcanes. The tissue cultured plants possessed a higher number of nodes with a higher internode distance. On the contrary, the periphery of the tissue cultured plants was smaller than that of offsets raised sugarcane plants (Sood et al, 2006)

Field performance in lingonberry (Vaccinium vitis) was compared (Gustavsson and Stanys, 2000). Fruit yield was significantly greater for tissue cultured plants than for stem cutting plants in both the second (79 %) and third (190 %) years, but mean fruit weight was not influenced by propagation method. Rhizome production and total plant weight were also greater for the tissue cultured lignoberry.

Seventeen clonal lines of Yucca valida were propagated and evaluated over a period of 26 months in an experimental plantation and compared with the performance of plants from seeds (Arce-Montoya et
The large variability found between the plants derived from seeds is manifested in the differences observed between the different clonal lines.

Tissue culture derived off-type plants with both good and poor field performance have been reported in banana (Msogoya et al, 2011). The study was conducted to evaluate the performance of the in vitro derived off-type banana in comparison with the in vitro micropropagation (MP) derived normal banana and conventionally propagated (CP) banana. The off-type fruits were significantly firmer with higher dry matter content of 12.4 kg/cm² and 33.7 %. The firmness and dry matter content of the MP derived normal banana were 8.5 kg/cm² and 20.0 %, and those of the CP derived banana were 8.9 kg/cm² and 21.1 %, respectively. The off-type fruits had significantly longer shelf life of 17 days compared with 7.2 and 7.0 days of the MP and CP derived normal banana, respectively.

Field performance of *Theobroma cacao* propagated via somatic embryogenesis revealed that tissue cultured plants demonstrated normal phenotypes in field conditions and have growth parameters
similar to plants propagated by traditional methods (Maximova et al, 2008).

3.1. Performance of regulators (secondary metabolites) in field

Research in the area of plant tissue culture technology has resulted in the production of many pharmaceutical substances for new therapeutics. Advances in the area of cell cultures for the production of medicinal compounds has made possible the production of a wide variety of pharmaceuticals like alkaloids, terpenoids, steroids, saponins, phenolics, flavonoids, and amino acids. Plant tissue culture is found to be an attractive alternative approach to traditional methods of plantation as it offers a controlled supply of biochemicals independent of plant availability (Sajc et al, 2000).

In vitro raised shoots and roots of Centaurium pulchellum were found to contain secoiridoids and xanthones increases in metabolites when in vitro tissue was compared with tissue produced in natural growing plants (Krstic, 2003). Micro-rhizomes of Curcuma longa were found to produce secondary metabolites with antioxidant activity equaling or
surpassing commercial dried powdered rhizome preparations of field-grown plants (Cousins et al, 2007).

4. Photoautotrophic micropropagation
Carbon dioxide and light are important for growth and development of plantlets in vitro (During and Harst, 1996). In most tissue culture systems, sucrose is used as a major carbon and energy source (Thompson and Thorpe, 1987). Incorporation of sucrose in the culture medium increases the risk of contamination and forces the shoot cultures to develop heterotrophy or mixo-heterotrophy (Kozai et al, 1992; Gabriela, 2005). These in vitro conditions, often result in plants with abnormal morphology, anatomy, physiology and in some cases genetic and cytogenetic variation (Pospisilova et al, 1999) creating serious problems in acclimatization of plants during micropropagation. The goal of micropropagation is to obtain a large number of genetically and physiologically uniform plantlets with high photosynthetic potential (Solarova and Pospisilova, 1997). One way to achieve this is by photoautotrophic or photomixotrophic multiplication with high CO₂.
Autotrophic (sugar free) tissue culture system was studied for promoting the growth of *Limonium* plantlets *in vitro* for reducing biological contamination (Kozai, 1988). Autotrophic micropropagation was considered as a novel system (Kozai, 1989).

In complete absence of sucrose (when only CO\(_2\) was provided as carbon source) explants of tobacco (Solarova *et al.*, 1989; Ticha, 1996), *Wrightia tomentosa* (Vyas and Purohit, 2003) and *Chlophytum borivilianum* (Joshi *et al.*, 2009) deteriorated and died. The effect of the presence/absence of sugar on net photosynthetic rate (NPR) of *in vitro* grown *Solanum tuberosum* under CO\(_2\) enriched condition was reported (Nakayama *et al.*, 1991). The NPR, was 8-10 times greater in the sugar-free medium than in the sugar-containing medium but the increase in dry weight of the explants was 5 times greater in the sugar-containing medium than in the sugar-free medium.

(Solarova and Pospisilova, 1997) were well established. Photoautotrophic micropropagation of some tropical and subtropical woody plants including fruit species (mangosteen), industrial plant species (coffee) and some forest tree (Eucalyptus, Acacia, Pauwlonia, Neem and Gmelina) were also reported (Nguyen et al, 2001).

Considerable reduction in the production costs could be achieved in autotrophic/mixotrophic micropropagation by means of deleting sugar, preventing microbial contamination, promoting plantlet growth, humidity control, increasing environmental stress tolerance, increasing survival percentage (reducing vitrified plantlets), reducing mutation and reducing salt concentration of the medium (Kozai et al, 2002).

The growth of plantlets in vitro is often greater under photoautotrophic/photomixotrophic conditions than under heterotrophic conditions, provided that the in vitro environment is properly controlled for promoting photosynthesis (Kozai, 1991a).

In order to estimate the photosynthetic rates of the plantlets in the
closed vessels, a model of CO₂- net photosynthesis was developed (Fujiwara et al, 1987). The model is given by the following equation:

\[ P_n = P_{ns} [1 - \exp \{-GP_{ns}(K_C - C)\}] \]

Where,

- \( P_n \) - net photosynthetic rate [\( \mu \text{cm}^3 \text{ CO}_2 \text{ mg}^{-1} \text{ h}^{-1} \)],
- \( P_{ns} \) - saturated NPR for CO₂ concentration [\( \mu \text{cm}^3 \text{ CO}_2 \text{ mg}^{-1} \text{ h}^{-1} \)],
- \( G \) - gradient of CO₂-net photosynthesis curve at CO₂ compensation concentration [\( \mu \text{cm}^3 \text{ CO}_2 \text{ mg}^{-1} \text{ h}^{-1} \text{ vpm}^{-1} \)],
- \( K_C \) - CO₂ concentration in the closed vessel [vpm], and
- \( C \) - CO₂ compensation concentration [vpm].

For plants under photomixotrophic conditions, promotion of photosynthesis is the primary way to enhance the growth rate of the plantlets. Elevated CO₂ enhance the photosynthetic ability of the plants (Kozai et al, 1990a).

### 4.1. Impact of elevated CO₂ on photosynthesis and photosynthetic characteristics

Photosynthetic characteristics of coffee (Coffea arabusta) and sorghum plantlets in vitro in response to different CO₂ concentrations and light
intensities were assessed (Nguyen et al, 2001; Cousins et al, 2003). The photosynthetic rate increased with the increase in CO₂ concentration in coffee, sorghum and tomato (Nguyen et al, 2001; Cristea et al, 1999). Moreover photosynthetic photon flux (PPF) saturation point was increased with increasing CO₂ concentration inside the vessel (Nguyen et al, 1999; Cristea et al, 1999). The low CO₂ concentration, reduced the NPR of plantlets/seedlings (Brassica campestris) in the vessel, and thus affected their growth and development (Kozai et al, 1990b). Elevated CO₂, increased the rate of photosynthesis of young, fully expanded leaves of Lolium Perenne by 35–46 % and of whole plants by more than 50% (Ryle et al, 1992).

Ultratructural and biochemical development of leaf tissue and key elements in C₄ (photosynthesis, carbon isotope discrimination, and leaf anatomy) pathway was characterized in the leaf of Sorghum bicolor plants grown at elevated CO₂ (Cousins et al, 2003; Walting et al, 2000). Elevated CO₂ had no effect on the cell-specific localization of Rubisco or PEPC at any stage of leaf development, and the relative ratios of Rubisco to PEPC remained constant during leaf development of
sorghum. However, in the oldest tissue at the tip of the leaf, the total activities of Rubisco and PEPC were decreased under elevated CO₂ implying that C₄ photosynthetic tissue may acclimate to growth under elevated CO₂ (Cousins et al, 2003). Elevated CO₂, lower the carboxylation efficiency and the CO₂ saturation rate of photosynthesis. This was accompanied by a 49 % reduction in the PEPC content of leaves in the elevated CO₂-grown plants (Walting et al, 2000). The ratio of quantum yield of CO₂ fixation to PS II efficiency was lower in plants grown at elevated CO₂. Analysis of leaf sections of sorghum grown under elevated CO₂, indicated a 2-fold decrease in the thickness of the bundle sheath cell walls in plants grown at elevated relative to ambient CO₂ suggested significant acclimation to increased CO₂ concentrations (Walting et al, 2000). The photoautotrophically grown plantlets of Chrysanthamum showed better growth and multiplication, higher content of chlorophyll and carotenoids, higher chl a/b ratio, net photosynthetic rate and ribulose 1, 5 bi-phosphatocarboxylase/oxigenase and PEPC activities (Cristea et al, 1999).
Effect of elevated CO₂ on different growth parameters

Moreover, elevated CO₂ enhances the physical growth parameters (Number of shoots and roots, shoot length, root length, number of leaves, number of nodes etc) of the plants grown under controlled CO₂ envirnment.

The effect of photoautotrophic and photomixotrophic micropropagation on different growth parameters was studied (Rahman and Alsadon, 2007; Jeong et al, 1995; Cristea et al, 1999; Kozai et al, 1991b; Vyas and Purohit, 2003, Dave and Purohit 2004; Joshi et al, 2009). The Shoot length and fresh weight of potato plantlets were significantly higher under photomixotrophic conditions (Rahman and Alsadon, 2007) than photoautotrophic conditions. Whereas the number of nodes per shoot was not significantly different on media with or without sugar.

Fresh and dry weights, height, leaf area, and chlorophyll concentration significantly increased with increasing CO₂ concentration in Gerbera (Jeong et al, 1995). However, percent dry matter and number of leaves were not affected by CO₂ concentration in Gerbera but fresh and dry
mass of tomato was increased due to high photosynthetic rate during CO₂ enriched conditions (Stein et al, 1983). Chlorophyll concentration decreased significantly as air exchange rate increased.

Under elevated CO₂ *Fragaria ananassa* (Strawberry) plantlets showed increase in fresh and dry weight of plantlet, NPR, the number of unfolded leaves, and ion uptake of PO₄³⁻, NO₃⁻, Ca²⁺, Mg²⁺ and K⁺ ions.

The plantlet growth *Lymonium* and *Cymbidium* promoted considerably by CO₂ enrichment under high PPF and by CO₂ enriched treatments (Kozai et al, 1990a; Kozai et al, 1987). The dry weight of *Cymbidium* and strawberry increased 1.7 to 2.0 times greater in high PPF and CO₂ enriched conditions (Kozai and Sekimoto, 1988).

*In vitro* growth and shoot multiplication of *Wrightia tomentosa, Achras zapota* and *Chlorophytum borivilianum* was studied in a controlled carbon dioxide environment (Vyas and Purohit, 2003; Dave and Purohit, 2004; Joshi et al, 2009). Complete absence of carbon source caused severe yellowing of shoots and death within 15-30 days (Vyas and Purohit, 2003, Dave and Purohit, 2004). Under photomixotrophic
conditions, best response was obtained at 0.6 g m⁻³ CO₂ (*Wrightia tomentosa*), 10.0 g m⁻³ CO₂ (*Achras zapota*) and 40.0 g m⁻³ CO₂ (*Chlorophytum borivilianum*) in terms of fresh weight, dry weight and number of shoots.

The splints of micropropagated shoots of *Solanum tuberosum*, single node cuttings each with a leaf, were inserted directly into the rock wool plugs and cultured under controlled the environmental control units at different intensities of solar radiation with CO₂ non-enriched and CO₂ enriched conditions. High solar radiation and CO₂ enrichment promoted growth in terms of fresh and dry weight of plantlets and rooting of the plantlets in the direct *ex-vitro* rooting method (Hayashi *et al.*, 1990).

Under elevated CO₂ concentrations, the leaf area index of the main shoot increased in spring wheat. At anthesis, stem and ear dry weights and plant height were increased by 174 %, 5 % and 9 cm, respectively, and biomass at maturity was 23 % greater in the 680 ppm CO₂. Grain numbers per spikelet and per ear increased by 0.2 and 5 grains, respectively, and this, coupled with a higher number of ears bearing
tillers, increased grain yield by up to 33 % (Mulholland et al, 1997).

The CO₂ enrichment improves plantlet growth, survival percentage during hardening, acclimatization, shipment and transplantation and reduces the risk of contamination. Growth and multiplication of shoots in a CO₂ enriched environment and their subsequent hardening and acclimatization has been achieved in a number of cases (Mousseau, 1986; Fujiwara et al, 1988; Solarova and Pospisilova, 1997).

Photoautotrophic multiplication not only reduces the losses due to contamination and leads to development of plants which are able to acclimate quickly to decreased air humidity. The leaves of photoautotrophically grown cultures have lower stomatal index (Khan et al, 2003). Such plants would show higher survival rates and better growth (Langford and Wainwright, 1987). Improvement in survival during hardening and acclimatization of tissue culture plantlets has been achieved in number of herbaceous species through in vitro growth of shoots/plantlets under CO₂ enriched environment (Mousseau, 1986; Fujiwara et al, 1988; Solárová and Pospíšilová, 1997).
5. **RAPD analysis**

Molecular markers are now routinely used for characterization of genetic diversity, DNA fingerprinting, genome mapping, genome evolution, ecology, taxonomy, and plant breeding. DNA-based markers are abundant, highly polymorphic and independent of environment or tissue type. Most DNA-based markers can be classified into three categories depending on the technique used (Karp and Edwards, 1997): Hybridization-based DNA markers, arbitrarily primed PCR-based markers, and Sequence targeted and single locus DNA markers.

Restriction fragment length polymorphism (RFLP) is hybridization-based markers in which DNA polymorphism is detected by digesting DNA with restriction enzymes followed by DNA blotting and hybridizations with probes. Arbitrarily primed PCR-based markers are employed in organisms for which no genome sequence is available. These markers are RAPD and AFLP. On the other hand, STS, SSR and SNP markers belong to sequence targeted and single locus PCR-based DNA markers. In current study, we used RAPD (Williams *et al*, 1990) to determine genetic variation induced during tissue culture process.
RAPD has many advantages: non-radioactive detection, multiple loci detection in a single reaction, requirement of small quantity of DNA, no need of prior sequence information, quick, inexpensive and technical simplicity (Leroy et al, 2000).

5.1. Molecular Marker Studies in Tissue Cultured Plants
Variation in both morphology and genotype has been reported to occur during in vitro regeneration processes (Kaeppler et al, 2000). Molecular tools are more reliable than phenotypic observation for evaluating tissue culture induced variations (Leroy et al, 2000). There are reports indicating that dedifferentiation of plant tissues lead to genetic modifications (Taylor et al, 1995; Hashmi et al, 1997; Rani et al, 2000). On the contrary, several reports also confirmed genetic integrity of tissue culture derived plants (Dale et al, 1981; Haydu and Vasil, 1981; Hanna et al, 1989; Jayanthi and Mandal, 2001; Gagliardi et al, 2004).

In *Arachis retusa*, 90 genomic regions were observed using 5 random primers which generated an average of 18 loci per clone (Gagliardi et al, 2004). All RAPD loci were monomorphic in the plantlets
regenerated from both apical segments and embryo axes. A report on *Macadamia tetraphylla* revealed that RAPD analysis could establish the clonal integrity of tissue cultured generated plants (Mulwa and Bhalla, 2007). Polymorphism was not observed 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 micropropagated 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 regenerants. No variation was also observed among the regenerants of *in vitro* cultured *Bambusa balcooa* (Gillis *et al*, 2007). Another report on somaclonal variation in *Vanilla planifolia* (Reddampalli *et al*, 2007) reported genetic uniformity among the micropropagated plants within one genotype. No variation observed among the regenerants using both RAPD and ISSR markers. Rout *et al* (1997) studied the genetic fidelity
among *in vivo* and *in vitro* plant materials using RAPD markers but could not notice any variation among the micropropagated plants of *Zingiber officinale*.

RAPD analysis of somatic embryo-derived plants of *Tylophora indica* and *Picea mariana* was undertaken to determine genetic homogeneity and the true-to-type nature of the regenerants (Jayanthi and Mandal, 2001; Isabel *et al*, 1993). Shoyama *et al* (1997) suggested that somatic embryogenesis could be used for clonal propagation of *Panax notoginseng* as genetic homogeneity was found among all the micropropagated plants using RAPD markers. Twenty-one RAPD primers produced monomorphic bands all across the 17 regenerants produced through somatic embryogenesis.

The lack of variation in somatic embryo derived regenerants could be due to the stringent internal genetic controls throughout embryo formation causing selection pressure against abnormal types (Leroy *et al*, 2000). In contrast, several studies reported tissue culture induced variation in a variety of plant species. In *Gypsophila paniculata,*
detected a very low variation was at the DNA level among the intact plant and the regenerants using RAPD (Ray et al, 2006).

Complete uniformity among the regenerants and field grown mother clone was reported in Musa (Lakshmanan et al, 2007). On the other hand somaclonal variation was also observed in banana (Smith, 1988; Vuylsteke et al, 1991). This contradictory result could be explained as all in vitro conditions were same during micropropagation but genetic variation in a culture line could be affected by the genotype more than the period in culture (Vendrame et al, 1999).

These studies clearly demonstrated that DNA amplification techniques like RAPD could be used to detect somaclonal variation in different micropropagated plants as well as genetic diversity among the cultivars. In this study, we have use RAPD which has been proved to be a potential DNA marker to determine the genetic fidelity/variability among the regenerants.