Review of literature

2.1 Trend of work on micropropagation in pteridophytes

Pteridophytes are described as the most developed among the non flowering plants and about 280-230 million years ago they dominated the forest (Mehra 1967; Khare 1996). At present angiosperms are dominating plant but a good number of pteridophytes are found in the forest of India due to various climatic condition and geographical features(Kaur, 1979,1980).

Ferns are used as medicinal herbs, as vegetables and as ornamentals for beautification. Bir (1987a) estimated about 500 species of ferns and 100 species of fern allies are present in India. Bir (1987b, 1992) identified rare and endangered pteridophytes in different region of India.

Ferns have a great commercial value based upon the good ornamental qualities of foliage. Application of *in vitro* culture technique could contribute to increase production of sporophyte of those desirable species.Various author has been listed the economic value of ferns (Kaur,1989). Today the plant diversity is facing serious threats, due to habitat degradation, habitat loss and over exploitation of natural resources. Globally 30% of the flora is threatened (Raven,1999).

Earliest work on pteridiphytes culture was done by Steeves and Sussex (1952) on *Pteridium aquilinum* Var. Different aspect of spore germination of homosporous ferns was investigated by Nayar and Kaur (1968). Miller (1968) studied on gametophytes produced from spores. Various stages of spore germination were studied by Klekowski (1969). Dyer (1979) examined the experimental biology of fern and formulated medium for fern germination.

Protocol for clonal mass propagation of the fern *Criptomium falcatum* was established (Garcia and Furelli, 1987). Spores of *C. falcatum* was incubated in MS medium and investigated the role of various chemicals such as sodium phosphate, L- cystein, different concentration of auxin (NAA,IBA), cytokinin (kinetin) on the growth of sporophyte and observed that in presence of NAA BAP or kinetin in media enhance the tissue growth.

Maeda *et al.*,(1990) formulated the protocol for formation of gametophytic tissue from the protoplast of young sporophyte of *Lygodium japonicum.* Propagation of fern from spore is a reliable method and considered more advantageous than vegetative mode of propagation (Linsay, 1994). Deberg (1994) reported that tissue culture using spore germination methods permits spore to be free from contamination by the spore of other species as well as infection by bacteria fungi, algae and mosses.

Fay (1994) used calcium hypochlorite, mercuric chloride and H_2O_2 as a sterilants for tissue culture. Most popular media for spore germination was Knop (1865) and Knudson (1946) media. Carbohydrates were considered very critical for spores (Whittier, 1965).Exposure to light be important criteria for spore germination (Dyer, 1979). Whittier (1981) reported some species of fern require darkness to germinate. Growth and development of the prothallus was influenced by some chemical and physical factors such as light, pH, physical state of medium and plant growth regulators (Hotta and Osawa, 1958, Mohr, 1962, Kato,1964; Miller, 1968;Fernandez, 1996;1997a,1997b,).

Cheema (2005) cultured two aquatic ferns viz *Marsilea minuta* and *Ceraptoteris pteroides* and for that different media such as Knop's medium (half and full strength) Knudson's medium (1951),Moore's medium and Murashige and Skoog's medium (1962) was tried. Among all the medium tried Knop's medium was established as optimal basal medium for the growth of the sporophyte.

Salome *et al*.,(1987) cultured the fern *Adiantum capillusveneris* foliar leaves explants on Gamborg B_5 medium and best result was obtained when medium was supplemented with 0.5mg/L IBA and 0.01mg/L BA and 2% sucrose. For Root initiation shoots were transferred to the Gamborg B_5 medium containing 0.05mg/L NAA after six week.

Low concentration of micro and macro nutrients favours the spore germinationand early gametophyte development in *Cyathea australis*.(Goller and Rybczynski, 1995 *Dicksonia solviana* (Khoo and Thomas,1980). Shukla and Khare (2012b) worked with *Cyathea spinulosa* and observed that when media (Parker's and Thomson) was supplemented with 8.87µM BAP and

2.21µM 2,4-D induced Callusing and maximum shoot formation found in media with 4.52µM BAP and 5.36µM NAA. Root formation enhanced in the medium containing 2.24µM IBA.

Goller and Rybczynski (1995) with *Cyathea australis* and stated that they observed better result when leaves containing sori was sterilized with 3% chloroamine and Tween for 30 minutes.Tissue culture technique have been used to propagate many fern species such as *Asplenium nidus* (Khan *et al.*,2008), *Dryopteris affinis* (Soare *et al*.,2010), *Blechnum spicant* (Menendez *et al*., 2009). Only a few studies were reported on the genera *Cyathea* such as *C.dregei* (Finnie and Staden, 1987), and *C.spinulosa* (Shukla and Khare, 2012b). The *in vitro* culture was used in order to achieve the propagation of ornamental ferns (Fernandez and Revilla, 2003), of the endangered ferns (Renner and Randi, 2004).

Beck and Caponetti (1983) studied the effect of kinetin and NAA on *in vitro* shoot proliferation and root formation in the fishtail fern and data revealed that NAA was not essential for shoot formation and kinetin was the major factor which induces shoot multiplication and media containing $5x10^{-7}$and 10^{-6} M kinetin in absence of NAA induced rooting. Addition of sucrose to the medium enhanced growth rate but not induced sporophyte formation. Dilute MS medium (to 1/10, 1/20,1/40 or 1/80) was suitable for sporophyte production (Kuriyama *et al.*, 2004).

Half strength MS medium with 3.33µM BA and 2.32µM kinetin induced earlier and higher number of aposporous gametophytes (Martin *et al*., 2006). According to the report 1/10 strength MS medium without sucrose was superior for the induction of apogamy in silver fern and during this experiment they also reported about the effect of sucrose on sporophyte production. Number of sporophyte production increased with the presence of decreased concentration of sucrose. Sporophyte formation increased with the increased concentration of sucrose in the medium has been also reported in other fern species (Whittier and Steeves, 1960; Fernandez *et al*., 1996).Induction of sporophyte from megagametophyte was published (Mahlberg and Baldwin,

1975) in *Regnellidium diphyllum* and *Marsilea vestita*. Full strength MS medium was suitable for the development of sporophyte in *Dryopteris affinis.*

Large scale propagation of *Rumohra adiantiformis* (G. Frost) Ching was achieved by Chen and Read (1983). Prague's medium was used for tissue culture and reported that 10mg/L ip, 10mg/L kinetin or 1mg/L zeatin in the medium induced 20-25 fronds after 9 week of incubation. Frond development favoured when liquid or solid medium was supplemented with 0-0.2mg/L kinetin.

Growth of gametophyte and sporophyte development were not antagonistic events and no correlation was found between the proportion of sexual phenotypes and rate of sporophyte formation (Somer *et al.*,2008)

Khan *et al*.,(2008) cultured *Asplenium nidus* using spores as explants. Spores cultured on half strength MS medium produced prothalli after 12 weeks. Maximum number of shoot was obtained on media containing 2mg/L NAA and 0.5mg/L BAP with various concentration of sodium dihydrogen phosphate (NaH_2PO_4). Optimum rooting was obtained at 2.0 mg/L IBA. Presence of sodium dihydrogen phosphate in MS medium plays a significant role in inducing sporophytic stage from gametophytic stage.Hedge *et al*.,(2006) worked with *Drynaria quercifolia* and obtained morphogenetic callus on Knop's (1865) medium supplemented with 5mg/L 2,4,5-T on to solid or liquid media. They first formulated the protocol for induction of callus and sporophyte formation using the leafy structure of fern.

The effect of gibberellins and exogenous auxins on apogamy in *Dryopteris affinis* (Lowe) Fraser-Jenkins sp. *affinis* was studied by Menendez *et al*., (2006a). Apogamous sporophyte production was increased when MS medium was supplemented with NAA (0.53µM and 5.37µM) or GA_3 at 2.8µM.

Menendez *et al*.,(2006b) studied with *Blechnum spicant* and observed that spores when cultured on MS medium produce heart shaped female gametophyte and addition of BA in culture medium increased maleness. The age of the spore and sterilization effect the spore germination and gametophyte development of *Platycerium bifurcatum* (Camloh *et*

al.,1999).Multiple shoots were obtained from adventitious buds and leaf callus in *Ceropteris thalictroides* (Cheema and Sharma, 1994).Protoplast was isolated from *Salvania natans* and from cultured protoplast young sporophyte was obtained.

Shastri *et al.,* (2005) worked with *Pteris vittata* and cultured it by inducing callus. They developed a protocol for callus induction for an economically important fern *Pteris vittata* using leaf primordium as explants. They recorded that 2mg/L 2,4-D+0.5mg/L BAP was suitable for callusing.

Effect of sucrose concentration, agar concentration and media pH on the growth of Staghorn fern was investigated by Pevalek-Kozalina (1996). Fernandez *et al*., (1999) observed that for successful micropropagation of different ferns with different growth cycle required different requirements. He showed that homogenization of the gametophytes of fast growing and high sporophyte producing species produced hundreds of sporophyte within a small period of time but sporophyte production was inhibited by homogenization of gametophytes in *Osmunda regalis.*

Amoroso and Amoroso (1998) investigated the appropriate condition for spore germination and sporophyte development on some economic species of fern of Mindanao, Phillipines viz. *Platycerium grande, Asplenium nidus, Blechnum orientale, Cyathea contaminans, Pteris mutilata var. victorae, Adiantum capillus-veneris, Osmunda banksiifolia, Lygodium japonicum and Lygodium flexuosum.* Highest percentage of spore germination (94%-95%) after thirty days of inoculation was observed in three species viz. *Lygodium flexuosum, Asplenium nidus and Blechnum orientale*. All of the fern species developed sporophyte from gametophyte.

Elaine Cristina de Paula *et al.,*(1999) worked with *Dicksonia sellowiana* and after seven days recorded maximum percentage of spore germination at 23±2°C in white light. The higher percentage of spore germination was observed when duration of application of white light per day was higher. The spore germination percentage was lower when spores kept under 43% and 2% of full sunlight. After 731 days of storage (at 10°C) 82% spores germinated.

Kuriyama and Maeda (1999) worked with *Equisetum arvense* and showed direct sporophyte development from spores without the gametophytic stage in liquid MS medium with cytokinin.MS medium supplemented with cytokinin formed cell masses after germination and then developed into sporophytic plant.

Treyes *et al.,*(2001)cultured the spores of *Cyathea contaminans* to develop prothallus and protoplast was isolated from the immature prothallia which was used to develop antheridial structure. Zhang *et al.,*(2001) studied the morphologic changes during spore germination and gametophyte development under culture condition. Spore germinated after 4-5 days and uniseriate and multiseriate filament developed after 7-8 days, gametophytes developed after 20 days, antheridia and archegonia developed after 25 days and within 10 days of fertilization sporophyte start to develop as recorded by them.

Huang *et al.*,(2004) observed that size of gametophyte of *Osmunda cinnamomea* is related to the population density and it negatively related. Jiamin (2004) observed that within 7-9 days spores of *Pteris multifida* germinate and mature prothallus developed after 80-100 days of inoculation. The structures of antheridia are globular and structures of archegonia are common fern type.

Shengjun *et al*., (2006) worked with *Sphaeropteris hainanensis* and reported that germination time of the spores in the sporangiums decrease from 20 days to 9 days when the sporangiums were pretreated with 50mg/L GA_3 for 5 minutes. Required time for spore germination was reduced 7days to 8 days when spores cultured on medium containing low concentration of NAA, KIN, BA. Media containing 2.0mg/L BA, 0.1mg/L NAA and 5g/L agar was efficient for prothallus development.

Mehra and Sulklyan (1969) cultured the gametophytes of *Ampelopteris prolifera* on mineral nutrients supplemented with various concentrations of sucrose to induce apogamy. Higher concentrations (5.8%) of sucrose were detrimental to prothallial growth while the lower concentrations (2-3%) delayed the apogamy.

Spores cultured on media with 2,4-D(3-5mg/L) produced gametophytic callus. Differentiation of rhizome segment into gametophytes and sporophytes was conditioned by the length of rhizome segments and sucrose concentration of the medium.

Ganguly *et al*., (2009) studied the gametophytic development of *Arthromeris himalayensis*. Spores germinated after 9-10 days of inoculation. Archegonial and antheridial phase developed on separate thalli.

Yin-li *et al*.,(2009) investigated the effect of different sterilization methods, medium and light intensity on propagation of *Dryneria roosii*. 49% spores germinated in Knop's medium. Spores germinated within 6-7 days after inoculation and after 100 days sporophytes developed. For sterilization 0.5% NaClO for 5 minutes was used and inoculated in half strength MS medium. Spores germinated in dark but failed to develop prothallial structure.

Mazumder *et al.*, (2010a) cultured *Bolbitis costata* and recorded the effect of growth regulators on *in vitro* propagation. Maximum germination percentage was observed on MS medium with 0.2mg/L IAA. Gametophytes cultured on MS+0.4mg/L IAA+ 5mg/L KIN + 0.2 mg/L IBA developed maximum sporophytes. Mazumder *et al.,*(2010b) cultured the spores of *Helminthostachys zeylanica* on Parker and Thompson basic Fern medium and MS medium. Maximum germination percentage was found in Parker and Thompson medium supplemented with 0.2mg/L IAA without sucrose.

Soare *et al*., (2010) reported the regeneration of *Dryneria affinis* and for that sporangia were inoculated on half strength MS medium without hormone which formed embryo after 45 days of inoculation when cultured explants were incubated at 25±1°C, 16 hour photoperiod and8 hours of darkness. Cultured plants were transplanted into the soil after the 10 month of spore inoculation.

Behera *et al*., (2011) observed spore germination within 10 days of spore inoculation of *Dipteris wallichii* (R.Br.ex Hook.et Grev.)T. Moore on Parker and Thompson's media. Archegonia development on gametophyte was

recorded on 49th day and on 55th day antheridia development was recorded. Sporophytes developed on 105th day of inoculation.

Singh *et al*., (2012) worked with *Anaemia rotundifolia* and cultured apical part of gametophyte on Parker and Thompson's medium which produce secondary regenerants with archegonia and from secondary regenerants tertiary gametophyte originated which bear male and female gametes and ultimately produced multiple sporophytes.

Less mortality rate and higher spore germination was listed by Marimuthu and Manickam (2011) when spores of *Pronephrium triphyllum* and *Sphaerostephanos unitus* was treated with 0.1% mercuric chloride for 3-5 minutes and rinsed with sterile distilled water for 15 minutes. The highest percentage of spore germination (36.8±1.31) in *S. unitus* and sporophyte formation (76.8±1.41) in Knop's medium was observed. In case of *P.triphyllum* the highest percentage of spore germination (38.3±1.13) and sporophyte formation (76.8±1.41) in Knop's medium.

Spores of *Dryneria quercifolia* germinate on full strength MS medium containing 1.0mg/L, 5.0mg/L KIN, 20% coconut milk and 300 mg/L casein hydrolysate was observed (Mazumder *et al.,*2011). On full strength Parker and Thompson media with 5.0mg/L IAA and 2.0mg/L KIN was effective for sporophyte regeneration and rhizome proliferation.

Kwa *et al*., (1988) reported that fronds of *Drymoglossum piloselloides* in MS medium with 1% agar produced aposporous gametophytein about 2 month. The presence of kinetin in the medium enhanced the ability of older fronds to develop aposporous gametophytes. Young fronds have a greater ability to develop aposporous gametophytes than the older fronds.

Camloh *et al*., (1994) used leaf of Platycerium bifurcatum to develop adventitious buds in MS medium with 5 or 10µM BA. Media with 6µM IBA showed optimal effect on rooting. Cheema and Sharma (1994) worked with Ceratopteris thalictroides and used adventitious buds and leaf callus to develop multiple shoots.

Shukla and Khare (2012a) obtained profuse callusing (80%) on full strength MS medium with 2.26µM 2,4-D + 2.22µM BAP using leaf primordium explants of *Pteris vittata*. Explants cultured on ¼ Parker and Thompson medium with 4.44µM BAP and 2.68µM NAA developed shoot (2.8±0.06) from calli.

Somatic embryogenesis and *in vitro* culture of *Huperzia selago* shoots was achieved by Szypula *et al*.,(2005) on ½ strength MS medium mineral salt content and also determined the difference of huperzine A content of *H. selago* shoots grown *in vitro* and natural habitat. Highest percentage of huperzine A content (3.33mg) found in shoots of plants grown from somatic embryos and differed from 0.54mg to 1.75mg in plants from natural habitat as demonstrated by HPLC- UV assay.

Thakur *et al*., (1998) used meristem of *Matteuccia struthiopteris* for *in vitro* culture and achieved high rate of multiplication on MS supplemented with 2.0mg/LN-(4-pyridyl)-N-phenylurea(4-pu) and 0.5mg/L thidiazuron (TDZ). They reported that suspension culture was more efficient in terms of multiplication of shoot primordial than solid medium. ¼ MS medium with 0.4%agar and 1.0% activated charcoal was efficient for growth of regenerants and rhizoid induction. Dykeman and Cumming (1985) and Hicks and Von Aderkas (1986) used solid media for *in vitro* culture of *M. struthiopteris.*

Loescher and Albrecht (1979) cultured *Nephrolepis exaltata var.bostoniensis* using the tissues of runners. Atmane *et al.*, (2000) formulated a effective protocol for *in vitro* regeneration of *Lycopodiella inundata* (Marsh clubmoss), a endangered medicinal Lycopod by using medium containing a few minerals and organic compounds supplemented with 0.05µM IBA and 1.4µM KIN and when calluses were cultured on medium with 2.5µM IBA+ 0.33µM GA3 plantlets developed from callus.

The diversity of forms of ferns is enormous and they thrive in many habitats.None of these primitive plants produce either seeds or flowers and they reproduce via single celled spores. Pteridophytes are peculiar in having alternation of generations with independent gametophyte and sporophyte phases. Sporophytic phase is of commercial importance for its ornamental,

ethnobotanical and medicinal properties. Ferns were the dominant part of the vegetation during carboniferous period (the age of ferns).*Cyathea gigantea* is a giant fern and it is widely distributed in hill region of eastern and peninsular India (Jain and Sastry,1980).Jamir & Rao (1988) reported that it grows in all the states upto 1200m altitude, in North-eastern region. During the recent years, *C. gigantea* is depleting at an alarming rate and presently the species is nowhere abundant or frequent as reported by Baishya and Rao (1982) and this species is treated as one of the endangered plants of India (Beniwal,1994).

Tree ferns are all true ferns in that they are flowerless plants and reproduce by spores. The spores are developed in sporangia on the underside of the leaves (Braggings *et al* . ,2004). In 2002, M. L. Khan *et al* ., published an article entitled " A plea for conservation of threatened tree fern (*Cyathea gigantea)*" in which they expressed their concern that especially this tree is on the verge of being extinct in and around the Itanagar (Arunachal Pradesh) and they also reported that the spores of *Cyathea gigantea* becomes fertile in February when forest floor is dry due to absence of rain which hindered the spore germination in exposed and deforested area and which is a major constraint for regeneration of new plant.

Ferns have been with us for more than 300 million years but in the world they are mostly neglected plants. *Cyathea gigantea* Wall. ex. Hook is a tree fern and have several active contituents like triterpenes, sterols, saponins, flavonoides,β-sitostenone and oleanolic acid (Juneja *et al.,*1990).Presence of flavonoid constituent in the genus *Cyathea* was reported by Harda and Saiki(1995). Oleanolic acid having anti tumor, hepatoprotective and antiviral activity (Wolska *et al.,* 2010, Zhou *et al*.,2011). Oleanolic acid found to exhibit strong anti HIV activity (Bhutani *et al*., 2010). β-sitosterol component of *Cyathea gigantea* have anti cancer activity(Woyengo, 2009). Traditionally the fresh rhizome of *Cyathea gigantea* mixed with black pepper seeds, powdered and taken orally with milk twice a day for one week against white discharges.(Rout *et al*.,2009).Chowdhery and Murti (2000) described *Cyathea gigantea* Wall. ex. Hook. as an endangered pteridophyte.

Naturally *C. gigantea* become fertile in February when forest floor is due to absence of rain which hindered the spore germination and it is a major constraint for regeneration of new plant. This tree fern species is an endangered plant with a number of medicinal importances but there is no published work in the field of tissue culture on *Cyathea gigantea*. Feeling the need of formulting strategies to conserve this species, *Cyathea gigantea* Wall. ex. Hook. is selected for *in vitro* propagation to frame a standard protocol.

2.2 Trend of work on micropropagation in genus *Dioscorea*

The monocotyledonous *Dioscorea* is known as yam. The genus *Dioscorea* includes 600 Species and is of considerable economic importance (Ayensu,1972). Many wild *Dioscorea* species, are a very good source of secondary metabolites which are used in pharmaceutical industry and medicine and an important part of it. A large number of wild species of *Dioscorea* are source of compounds used in synthesis of sex hormones and corticosteroids (Coursey,1967) and cultivated species are the source of food in some countries (Coursey,1976). Root and tuber crops are the most important food crops and place the crops after cereals. Tuber crops provide food security to tribal population and thus make an important position in food habits.A rich genetic diversity of tuber crop are found in India especially yam *Dioscorea* (Hann,1995).

Yams are valuable source of carbohydrates, fibers and low level of fats which make them a good dietary nutrient and also processed into various staple intermediate and end product forms (Jallel *et al*.,2007). Many species of *Dioscorea* genus are economically important crops worldwide. eg. *D. alata, D. Cayenensis, D. rotundata* are main crops in Caribbean Central and South America and West Africa (Tor *et al*., 1998).*Dioscorea alata* L. provide food security to tribal peoples.

The tubers of some species of *Dioscorea* are important source of diosgenin, a chemical used for the commercial synthesis of sex hormones and corticosteroids which are widely used for anti-inflammatory, androgenic and

contraceptive drugs (Satour *et al*., 2007). Out of six hundred species of *Dioscorea* 14 are used as edible tubers. Tubers have a dual agricultural function. They supply nourishment as a source of food and tubers are also act as a planting material (Craufurd *et al*., 2006). *Dioscorea* species are vegetatively propagated by using tuber pieces. The production of tuber is hampered by several significant virus and fungal diseases (saleil *et al*., 1990).

Micropropagation of *Dioscorea* has been achieved through rapid proliferation of shoot-tips axillary buds in culture. Several factors were reported to influence the growth of *in vitro* propagated plants. Different explants were used by different worker to propagate their plant.

Mahesh *et al*.,(2010) tried to propagate *Dioscorea wightii* plant using nodal segment as explants. BA and kinetin was used for the proliferation of nodal segment. Callus formation was observed in MS medium supplemented with 0.15-1.75µM BA, 0.75-5.0µM kinetin, 0.15-0.30 µM 2iP and shooting was observed in all growth regulators tested in BA, Kinetin and 2iP. Ondo *et al.,* (2009) reported that presence of kinetin (2mg/L) reduce the shoot length, root length, node numbers but root length was increase when sucrose concentration increased 3%-5% in case of D. *cayenensis -D. rotundata* complex. It is observed that cytokinin required in optimal quantity for shoot proliferation in many genotypes but addition of low concentration of auxins along with cytokinins triggered the shoot proliferation (Sengupta *et al*.,1984). Kadota and Niimi (2004) reported that in terms of shoot proliferation liquid medium was most effective to solid medium , 6.9 number of node produced in liquid medium where 2.1 node produced in solid medium in *Dioscorea japonica.* 6-Benzylaminopurine at 0.44 µM produced highest number of nodes (7), shoot (2.60) and the fresh weight 336.0mg.40 ml LS medium supplemented with 0.44µMBAand 0.44µM NAA with no gelling agents was optimum for shoot proliferation. Jova *et al*.,(2005) investigated the effect of temporary immersion system on formation of micro tuber in *Dioscorea alata* and reported TIS show a positive effect on shoot growth.

Chen *et al*.**,** (2003) developed a protocol for rapid *in vitro* propagation of *D.zingiberensis* using stem as explants. Medium supplemented with 4.4µM

BAP+1.1 µM NAA produced shoots on nodal segments within 20 days. Callus formed on MS +8.9 µM BA+ 5.4 µM NAA in 30 days, 22.2 µM BAP and 1.1 µM NAA regenerated shoot from callus and for rooting 4.9 µM IBA was used. Poornima and Ravishankar (2007)used nodal segments to propagate *D. Oppositifolia* and *D pentaphylla* and reported multiple shoots produced on MS medium with 8.8 µM BAP and 0.3% activated charcoal, rooting was observed in MS medium with 2.67 µM IBA and developed tuber on MS medium with 8.8µM BAP. Behera *et al*., (2009) carried out a work to regenerate plantlet of *D. Oppositifolia* where nodal segment was used as explants and culture it on MS medium supplemented with BAP and NAA. They reported best shoot proliferation was observed in MS medium + 2mg/L kinetin + 1.0 mg/L BAP + 0.5 mg/L NAA + 100 mg/L ascorbic acid where 90% explants showed proliferation and half strength MS supplemented with 2.0mg/L NAA found to be best for root formation. Mantell (1998) studied the association of microbes with tissue and cell cultures of tropical *Dioscorea* yams for that nodal segment were used as explants. Ondo *et al*., (2010) gave an account on effect of polyamines on *in vitro* tuber formation and development in *D. cayenensis-D.rotundata* complex and found that in presence of polyamines in culture medium accelerated tuber formation. Ondo *et al*., (2009) investigated the effect of reducing sugar on *in vitro* tuber formation and sprouting in yam (*D. cayenensis- D. rotundata* complex) and observed for earlier tuber formation 1% sucrose needed but it decrease the length and weight of tuber, tuber obtained 3% sucrose sprouted rapidly.

The individual effects of sucrose, plant growth regulators and basal salt medium formulations on microtuber induction and development were investigated by Alizadeh *et al.,* (1998) and reported that BA at 1.25 and 2.5 µM strong inhibitory effects on microtuber induction while promotive effect was shown by NAA and IBA at 5.0 µM.

In vitro regeneration and multiplication of *Dioscorea alata* L. was studied by Borges *et al*.,(2004) and high rates (100%) of explant regeneration was observed in D-571 medium with 1.5%manitol +1mg/L BAP + 2g/L activated charcoal. An improved method of *in vitro* propagation of *D. bulbifera* was established by Forsyth and Staden (1982) for this they cultured nodal segment

on MS medium and reported that with the increasing concentration kinetin shoot formation per node was increased. Primary callus was induced by Shu *et al*.,(2005) culturing stems, leaves, petioles on MS medium supplemented with 0.5-2.0mg/L BA + 0-2.0mg/L NAA and best callus formation was observed in medium with 0.5mg/L BA + 2.0mg/L 2,4-D from stem explants. Chu *et al*.,(2002) cultured *Dioscorea* species in different day length, different concentration of BAP and sucrose and accumulation of soluble carbohydrate was found in leaves with the increasing concentration of BAP (0-22 µM/L)and sucrose(1.5-8%). Bazabakana *et al*.,(1999) investigated the effects of applying exogenous jasmonic acid (JA) on the microtuber germination of *D. alata* and listed JA at concentration (0.1-1 µM) promoted the germination but JA at concentration 30 and 100 µM completely inhibited the germination. Lauzer *et al*.,(1992)noticed that nodal segment less than 5 cm length was less suitable for *in vitro* propagation when worked with *D. abyssinica* and *D. mangenotiana*. Wheatley (2003) designed a experiment to develop salt tolerant yam (*D. alata*) and observed higher level of NaCl (200 mM) show devastating effect on shoot proliferation as well as root development, at concentration 100 mM NaCl development of new node and leaves formation was noticed.

Yan e*t al.*,(2011) studied the effect of temporary immersion system on growth and quality of *D. fordii* and *D.alata* and results reported by them indicated that TIS improved the growth and quality of the plantlets in terms of proliferation rate ,shoot length, fresh weight, dry weight of shoot and biomass. Nodal segments were used as explants by them. Highest rate of shoot proliferation was observed on MS medium with 2.0mg/L Kn+1.0mg/L BAP+ 0.5 mg/L (Behera *et al*.,2009). Nodal culture without growth regulators has been shown to be an efficient way for multiplication of several species of *Dioscorea* (Okezie,1987).

Heping *et al*.,(2008) worked with *D. zingiberensis* and for callus induction seeds were cultured on MS medium + 1.0mg/L BAP + 0.5mg/L IAA. Plantlets regenerated on solid MS medium with 0.2mg /L BAP and half MS medium with 0.5 mg/L NAA favoured root formation in regenerated shoots.

Effect of sodium nitroprusside on callus induction and plant regeneration was investigated by Xu *et al*.,(2009) in *D. opposita* and noticed supplementation

with 40 µM SNP markedly promotes callus induction frequency, higher number of shoots produced in SNP supplemented medium than the medium without SNP. Callus was induced from seed (Savikin-Fodulovic *et al*.,1998) on MS medium supplemented with 5mg/L 2,4-D + 0.5mg/L BAP for 5days and later they lowered the concentration at 0.66mg/L. Heping *et al*.,(2008)produced tetraploid plants of *D. zingiberensis* using seed by colchicines.

Inflorescence induction and morphogenesis of regenerated flowers was investigated in *D. zingiberensis* (Huang *et al.,*2009) and for that experiment male inflorescence was used as explants. According to the observation MS + 2mg/L BA + 0.5mg/L BA was favourable for highest inflorescence induction where GA showed reverse effect when kinetin combined with 0.4mg/L NAA explants developed inflorescence.

Ondo *et al.*,(worked with *D. cayenensis - D.rotundata* complex and reported jasmonic acid (JA 10µM) increase the tuber formation in absence of kinetin. *In vitro* production of microtubers has been reported in a number of species (Ondo *et al*., 2007; Ng,1988;Borthakur and Singh, 2002; Gibson, 2005).Jasik and Mantell (2000) reported media supplemented with 20g/L sucrose produce higher micro tuber number and greater micro tuber size than 40g/l sucrose. A decrease in the percentage of microtuberization with 8 and 10% sucrose and 2.5 µM kinetin in *D.rotundata* was reported by Ng (1988). Higher level of kinetin (23.2-46.4 µM) raised the microtuber formation frequency was reported by Forsyth and Van Staden (1982).Influence photoperiod on *in vitro* tuber formation was examined by Jean and Cappadocia(1991) and indicated 16 and 24 hour photoperiod was favorable to produce highest number of microtubers whereas 8 hour photoperiod was effective larger micro tubers. Mantell *et al.*,(1978) observed 2% sucrose produce maximum number of micro tuber in nodal culture of *D. opposita* and *D. alata*. For tuber growth increasing amount sugar is required in the medium (Jean and Cappadocia,1992).

MS medium has been reported to be inhibitory in case of tuber formation (Mantell and Hugo,1989).Highest number (2.2±0.14) of micro tubers were obtained on MS + 30g/L sucrose +2.0mg/L KN+ 1.0mg/L BAP+0.5mg/L

NAA (Behera *et al*.,2009).Kohmura *et al*.,(1995) studied the effect of sucrose concentrations (3 and 6%) in *D.opposita* with 8.0 µM BAP and they reported 6% sucrose was found to be more effective in tuber formation. Chen *et al*.,(2007) indicated sucrose provide a carbon source and energy for induction of shoot and micro tuber. Lauzer *et al.*,(1992) reported that under 8h day length in both species *D.abyssinica* and *D.mangenotiana* microtuber was induced on nodal segment. In *D. abyssinica* microtubers induced when sucrose was present in the medium at concentration 20, 40, 60 and 80 g/L whereas in *D. mangenotiana* tuberization favored only at concentration 40 and 60g/L.

Behera *et al.*,(2008) Used NAA and IBA to induce rooting from *in vitro* raised shootlets of *D.hispida*, and they observe highest rooting on half strength MS basal medium + 2mg/L NAA+ 2g/L Ac and 2mg/L IBA + 2g/L Ac in half strength MS basal medium induce second highest rooting. For rooting Behera *et al.*,(2009) *in vitro* micro shootlets of *D.oppositifolia* inoculated on half MS medium supplemented with 2mg/L NAA and profuse rooting was observed on this medium. 2mg/l NAA in combination with 0.2 and 0.5 mg/L BA produced root in *D. esculenta* (Belarmini and Rosario,1991). Poornima and Ravishankar (2007) reported that efficient rooting was observed on MS medium +2.67µM NAA after 30 days.

Sucrose concentration when raised 3% to 8% an increase in root number was observed (Ondo *et al*.,2007), sucrose concentration when increased 3% to 5% root length also increased. Rooting frequency was higher in the solid medium but number of roots produced by each shoots was greater in liquid medium and the roots produced in gellan gum medium was longest(Kadota and Niimi,2004). In hormone free medium within 10 days all the shoots produced root, when medium was supplemented with 4.9µM or 9.8µM IBA induced fastest rooting with higher number of roots per plant was observed (Chen *et al*.,2003).

Kadota and Niimi (2004) reported that when micropropagated plants of *D. japonica* were transferred to pots containing 1:1vermiculite and soil (v/v) mixture under green house condition about 80% of the plants survived. Micropropagated plants were transferred to the pots containing mixture of soil + sand + manure in 1:1:1 ratio (Behera *et al*., 2009) and 90% plants survived.

Rooted plantlets were transferred to the pots containing sand, compost and mould mixture (1:1:2), after 8 month acclimatized plants produced tuber (Bazabakana,1999).Rooted plantlets were transferred to soil rite (equal proportion of decomposed coir and peat moss) for acclimatization (Ondo *et al*.,2009).

Dioscorea alata L. is used as a staple food for millions of peoples living in tropical and subtropical countries and it is an important tuber crop (Edison *et al*., 2006).*Dioscorea alata* used as a treatment for various diseases like fever, leprosy, tumors, gonorrhea and inflamed hemorrhoids in folk medicine and this plant also used as vermifuge and laxative (http://en.wikipedia. org/wiki/Dioscorea alata).Yam propagation by seeds using conventional methods is slow and not adequate for rapid multiplication. Tuber yield is drastically reduced by viral and nematode infections; through infected tubers it is transmitted to the next generation (Ng, 1992) and it also deteriorate the quality of the tuber (Mitchell and Ahmed, 1999). *In vitro* propagation may help to overcome constraints related with availability of high quality of planting material (Wheatley *et al*., 2005; Vaillant *et al*., 2005).

Dioscorea species are vegetatively propagated by using tuber pieces. The production of tuber is hampered by several significant virus and fungal diseases (saleil *et al*., 1990). Choudhury *et al*., (2002) reportd this plant as an endangered plant so the plant species is selected for *in vitro* propagation.

In present investigation an attempt was made to cultivate *Dioscorea alata* L. *in vitro* using minimum number of growth regulators, so that the plant can be regenerated in mass for general use as well as commercial exploitation in minimum cost. Effect of indole acetic acid (IAA) on shoot and root initiation was also studied. It was noticed from review of literature, earlier worker propagated *D. alata* (Borges *et al*., 2009) using growth regulators kinetin, BAP, NAA, IBA and sucrose but effect of IAA was not investigated so this work was done to explore this ground.Media used for this experiment without sugar supplementation.

2.3 Trend of work on micropropagation in Orchids

Among angiosperms orchidaceae is one of the largest families. Orchidaceae family includes 800 genera and 25000 species (Stewart and Griffiths, 1995).Orchids are well known for their economic importance and ornamental beauty.Among monocotyledons orchids are the highly evolved family with near about 1000 genera and 25000-35000 species with diverse range in size, shape and colour of their flowers (Atwood,1986;Chadha,1992). In the world of ornamental plants orchid constitute an order of royalty.Orchids have immense importance in horticultural field and play a very useful role in balanceing the forest ecosystems (Kaushik, 1983). They are the most pampered plant and among all flowering plants it occupies the top position for its commercial value.Orchidaceae is one of the most morphologically as well as ecologically diverse families of flowering plants and it is the second largest family of flowering plants in the world, comprises of about 779 genera and 22500 species (Mabberley,2008).

India is considered as rich heritage of orchid and recognized as a significant producer of wild orchid in the world.1331species belonging to 186 genera of orchids are available in India (Misra, 2007).Nearly 300 species belonging to 75 genera are endemic to this country (Rao, 1991).North Eastern region of India has about 870 orchid species in 151 genera which constitutes nearly 70% of total orchid of our country (Jain, 1985). Orchids are a group of plants which grow in a different habitats (some terrestrial, some epiphytic) throughout the globe but they are very sensitive to changing habitat. A number of orchid species are rare and threatened through out the world.Threats to orchid species in the Indian region were first revealed by Pradhan (1971).

Orchids are grown for their ornamental beauty and are valued as cut flowers not only for their exotic beauty but also for their long shelf life.A single orchid capsule contains millions of seeds, which do not have any endosperm. A large number of seeds produced in capsule but only few seeds able to germinate in nature. Horticultural trade depends on wild orchid population as a source of stock plants so an artificial means of propagation is in demand to reduce the pressure on wild plant.

At present the orchids occupy a prominent place in the Red Data Book prepared by IUCN. So an efficient strategy needs to be designed to save this beautiful member of plant kingdom. Wimber (1963) published first a protocol for micropropagation of *Cymbidium* using meristem as explants. Geetha and Shetty (2000) developed a protocol for *Vanilla planifolia* for large scale micropropagation using shoot tips and nodal buds. This micropropagation technique utilizes two media first one MS+ 1mg/L BAP+ 3% sucrose in which explants first cultured and second one is N69 (Nitsch basal medium; Nitsch 1969) +0.5mg/LBAP +0.5mg/Lbiotin +0.5mg/L folic acid and 2% sucrose in which proliferating shoots are obtained. One simple protocol using MS+ 1mg/L BAP + 150mg/L CW was established (Kalimuthu *et al*., 2006) for initiation, multiplication, elongation and rooting of *Vanilla* .

Utilizing shoot tips of *Dendrobium chrysotoxum* as a explant embryogenic callus was obtained by Roy *et al*.,(2007) on MS+2µM TDZ/BAP. Seeni & Latha(1992) reported the successful regeneration of large number of plants from leaf tissue culture of endangered *Renanthera imschootiana* Rolfe. Chen *et al*., (1999) reported direct somatic embryogenesis from young leaves of *Oncidium* on half strength MS medium with 0.3-3mg/L TDZ. TDZ promote direct somatic embryogenesis from the epidermal cells and secondary somatic embryogenesis from leaf explants of *Phalaenopsis amabilis* has been reported (Chen and Chang, 2006). Jheng *et al*., (2006) reported that *in vitro* culture of *Oncidium* was better on MS with maltose than sucrose because highest number of PLBs formed on MS medium with maltose from 1g callus and after 4 month half of the PLBs transformed into plant on MS medium with 20g/L trehalose.

Rotor (1949) initiated *in vitro* culture of *Phalaenopsis* using flower stalks as a explants. Lin (1986) reported that developmental stage and age of flower stalk effect the frequency of PLBs formation during *in vitro* culture of *Phalaenosis* and *Doritaenopsis.* According to the report of Ernst (1994), TDZ alone in the culture medium has a promotory effect on miicropropagation of hybrids *Phalaenopsis* Dora and *Doritaenopsis* George Molar using inflorescence axis section.

Application of exogenous plant growth regulators is not indispensable for normal plantlet production of *Cymbidium* species as reported by Shimasaki and Uemoto (1990). Chang and Chang (2000) developed a protocol for plantlet regeneration of *Cymbidium sinense* Willd. using TDZ (0.001-1mg/L) from 1 cm apical segment of rhizomes. Paek and Yeung (1991) established the fact that cytokinins are effective in induction of shoot from rhizome segment of *Cymbidium forrestii.*

NAA are effective to induce rhizome formation and branching of many orchids like *C.kanran* Makino in culture (Shimasaki & Uemoto, 1990).Tanaka *et al*.,(1976) formulated a protocol for inducing PLBs using root tips of *P. amabilis.* Philip and Nainar (1986) established a method for rapid clonal propagation of *V.planifolia* Andr.on MS medium with 2mg/L IAA and 0.2mg/L KN using root tip and after 9 months 5-40 plantlets were formed from each root tip.

A large number of additives like peptone, carrot juice, tomato juice, beef extract, potato extract and coconut water, banana extract are commonly added to media used in orchid propagation. Beneficial effect of these additives have been reported in many orchid species such as *Aranda* Deborah (Goh and Wong, 1990), *V. coerulea* (Seeni and Latha, 2000), *V. spathulata* (Decruse *et al*.,2003), *Dendrobium tosaense* (Lo *et al*.,2004).

Malabadi *et al.*,(2005) cultured *Dendrobium nobile* on medium of Mitra *et al*.,(1976)with triacontanol (TRIA). Highest shoot proliferation was achieved media with 4.0µM TRIA and maximum root proliferation was observed on media with 2.0µM TRIA.

Chung *et al.*, (2005) examined the effect of four auxin IAA, IBA, NAA, 2,4-D and five cytokinin 2ip, BA, Kinetin, TDZ, Zeatin on direct embryo formation from leaf explants of *Dendrobium* cv. Chiengmai Pink cultured on half MS medium.18.16µM TDZ cultured in light for 60 days was recommended as the best condition for direct embryo formation as it formed 33.6 (mean number) embryos per explant in 33% of explants.

A rapid clonal propagation technique was developed (George and Ravishankar, 1997) for *Vanilla panifolia* using semi solid MS medium supplemented with 2mg/L BA and 1mg/L NAA from axillary bud.After 2-3 weeks of incubation multiple shoots were formed in MS medium supplemented with 1mg/L BA + 0.5mg/L NAA.

Yan *et al*.,(2006) established an efficient method for *in vitro* propagation of *Cypripedium flavum* using Havais media. Havais media supplemented with 2.22µM BAP and potato homogenate (20g/L) was effective in case of shoot multiplication frequency (95%) and produced maximum number of shoots (2.55 shoots/plant). Half Havais media supplemented with 0.6g/L activated charcoal was recommended as the best composition for rooting.

Martin (2007) formulated a protocol for micropropagation of *Arundina graminifolia* using nodal segments. Explants were cultured on half strength MS media with 6.97µM kinetin or 15% coconut water or 13.3% BA for axillary bud sprouting and then it was cultured on MS medium with 44.4 µM BA which facilitate PLBs formation. MS medium containing 6.97µM KN was effective for conversion of PLBs to shoots and for root initiation half strength MS medium supplemented with 1g/L activated charcoal was effective.

More than any other plant family orchidaceae have a large number of threatened species. North Eastern part of our country is the sweet home for many medicinal and ornamental orchids (Mehdi and Chakraborti, 2009).Among the total number of orchid species two- third of species are epiphytes and lithophytes and remaining third holding the terrestrial orchid. According to the report of World Coservation Union (IUCN1999) half of the total extinct orchids are terrestrial, herbaceous perennials. Terrestrial orchids experience a greater extinction risk due to multiplicity of threatening processes especially under present climatic change scenarios (Nigel and Kingsley, 2009). Orchids produce capsule within it a large number of seeds are developed but unlike many other angiospermic plant seed endosperm is lacking in orchid seed (Arditti and Ghani, 2000, Batty *et al*, 2000).

Arundina graminifolia (D. Don) Hochr. is commonly known as bamboo orchid. Bamboo orchid is a terrestrial perennial orchid. According to the report

of Jain and Sastry (1980) *Arundina graminifolia* (D.Don) Hochr. is an endangered orchid. Zenghong *et al*.,(1993) described *Arundna graminifolia* (D. Don) Hochr. as an important medicinal plant.

By continuous literature survey it is highlighted that orchids are facing the risk of extinction particularly terrestrial orchids are experiencing special risk of extinction. *Arundina graminifolia* is a terrestrial herbaceous perenial orchid and an endangered species so the species attract my special attention. Not much work is done on this species regarding tissue culture aspect, it is the need of the hour to work with this species to formulate a standard protocol for *in vitro* propagation to conserve the species so this medicinally important as well as endangered plant species is selected for *in vitro* propagation.

2.4 Trend of work on micropropagation in Zingiberaceae

Family Zingiberaceae constitute a major group of rhizomatous medicinal and aromatic plants characterized by presence of volatile oils and oleoresins. The family Zingiberaceae comprises of about 50 genera and 1400 species (Hsuan *et al.*,1998). Generally the rhizomes and fruits are aromatic, tonic and stimulant and they have nutritive value. The family Zingiberaceae is a large, well- known and important monocot family.

Rahman *et al*.,(2005) designed a protocol for rapid clonal propagation of Black *galanga* L. and reported that explants (rhizome tip and lateral bud) cultured on MS medium supplemented with 10mg/L BA and 0.1 mg/L NAA within 3 weeks produced 2-3 shoot buds. 12.4 roots/microshoot developed on the modified MS medium with 0.2mg/L IBA was also recorded.

Villamor (2010) worked with *Zingiber officinale* Rosc. to investigate the effects of media strength and source of nitrogen on the growth of shoot and root of ginger. Results revealed that nitrogen in the form of KNO_3 significantly enhanced proliferation rate of ginger *in vitro* in full and half strength MS media. More leaves were produced when MS media supplemented with NH_4NO_3 or without KNO_3.

MS media when supplemented with 10µM BA with 5µM IAA or 5µM NAA induced high rate of shoot proliferation in *Zingiber spectabile* as reported by Faria and Illg (1995). 100% rooting was obtained in liquid or gelrite gelled medium supplemented with 5µM NAA or IAA. Palai *et al*.,(1997) examined the effect of different concentrations of BA and IAA on multiplication rate of *Zingiber officinale* and listed that rate of multiplication was higher in the medium containing BA (4.0-6.0mg/L), IAA (1.0-1.5mg/L) and 100 mg/L adenine sulfate. *In vitro* organogenesis via callus culture of *Zingiber officinale* was tried (Rout and Das, 1997). Shoot regeneration was maximal in the MS medium supplemented with 5.0mg/L BA+ 1.0mg/L IAA+ 100mg/L of adenine sulfate and under continuous illumination shoot bud regeneration was highest as reported by workers.

Vincent *et al.*,(1992) propagated *Kaempferia galanga* L. *in vitro* using axillary meristem and nodal segments as explants. Authors reported that the highest number of shoots per explant was obtained on MS media supplemented with 0.50mg/L of BAP and 3.0mg/L Kinetin after 120 days of incubation. Guo and Zhang (2005) worked on *Zingiber officinale* Rosc. and used ginger shoot tip as explants and cultured the explants on MS medium containing 1.0mg/L 2,4-D and 0.2 mg/L KN which induced somatic embryogenic calli. When callus was transferred to MS + 0.2mg/L 2,4-D + 5.0mg/L BA + 3% sucrose + 0.7% agar somatic embryos produced roots and shoots and on MS + 3.0mg/L BA + 0.1mg/L NAA developed complete plantlets.

Faridah *et al*., (2011) micropropagated *Zingiber zerubet* Smith using rhizome buds as explants. Highest mean number of shoot per explants was produced on MS media with 5.0mg/L BAP and 2.0mg/L IAA or 3.0mg/L BAP + 0.5mg/L IAA. MS media containing 1.0mg/L BAP + 2.0 mg/L IAA induced highest shoot length and shoots cultured on medium with 5.0mg/L BAP + 2mg/L IAA induced highest number of roots and longest roots produced with 1.0mg/L BAP.

Swapna *et al.,* (2004) high frequency of organogenesis and multiple shoot formation were obtained on MS medium supplemented with 0.5mg/L IAA and

2.5mg/L BAP using rhizome as a explants and highest rooting was observed in MS medium with 0.5 mg/L IAA and 2 mg/L BAP.

Shirin *et al.*, (2000) investigated the effect of different concentration of BA and auxin in *in vitro* plantlet production of *Kaempferia galanga* using rhizome as explants. On 75% MS + 12µM BA + 3µM NAA and 3% sucrose *In vitro* plantlet production has been achieved. 100% plantlets survived after field transfer. Parida *et al*.,(2010) formulated a efficient protocol for rapid multiplication and *in vitro* leaf biomass in *Kaempferia galanga.* Different plant growth regulators was tried to induce multiple shoot using lateral bud of rhizome as explants. The highest rate of shoot multiplication shoot/explants (11.5±0.6) as well as leaf biomass production gram/ explants (7.4±3) was observed on MS media supplemented with 1mg/L BA and 0.5mg/L IAA and 95% plants survived when transferred to the field as recorded by author.

Kaempferia galanga and *Kaempferia rotundata* cultured *in vitro* on MS medium using rhizomatous buds as explants.In case of *Kaempferia galanga* multiple shoots were induced on MS medium containing 5.70µM IAA alone or in a combination of 0.57µM IAA with 4.65µM KN. Media supplemented with 2.69µM NAA + 2.22µM BAP was best for *Kaempferia rotundata* (Chirangini, 2005). After 3month of field transfer 80%-90%plantlets survived.

Bhatt *et al*., (2012) investigated the effect of different concentration sucrose, BA and photoperiod on *in vitro* propagation of *Kaempferia galanga*. They reported that MS + 5mg/L BA + 30 g/L sucrose and 4hour photoperiod induced highest shoot proliferation (7.4±1.0) shoots/explants and highest number of roots/shoot (31.3±3.2). Maximum shoot length (4.7±0.7 cm) and highest number of leaves/shoot (4.7±0.2) was obtained on MS medium containing 30g/L sucrose without BA and 16 hour of light. MS medium supplemented with 5mg/L BA and 30g/L sucrose 4 hour light or 20 hour dark photoperiod was selected for optimal condition for mass multiplication of *Kaempferia galanga.*

Mongkolchaipak *et al*.,(2006) cultured *kaempferia parviflora* and observed that optimal proliferation was on MS media supplemented with 7mg/L BAP and average 4.2 and 4.5 shoots produced after1 and 2 month respectively.

Maximum rooting was observed on solid MS with 1mg/L NAA. Survival percentage was recorded 98% and 96.5% after 1 and 2 month respectively.

Kaempferia parviflora Wall. Ex. Baker is a monocotyledonous plant belonging to family zingiberaceae. It is also known as black ginger. This plant species is very well known for its number of medicinal importance. Since ancient time it has been traditionally used as a health promoting and vitalizing agent (Yenjaj, 2004).

The rhizome of this plant used for leucorrhea, oral diseases, abdominal pain, health promotion and aphrodisiac traditionally (Wutythamawech, 1997).Chakma tribe for treatment of goiter and dysentry use the juice of rhizome of *Kaempferia parviflora* Wall. ex. Baker. Rhizome paste and leaf is used used for insect bite.Tanchangya tribe for the treatment of diarrhoea use rhizome juice. The plant is used by Marma tribe to stop bleeding from nostrils (www. mpbd.info/plants/Kaempferia parviflora.php).

Kaempferia parviflora Wall. ex. Baker is a herbaceous plant belonging to Zingiberaceae family (Sirirugsa,1992). Its black to purple rhizomes used as flavouring agent in local food and as a folklore medicinal plant for the treatment of a wide spectrum of illness. This plant is known in as Krachai-dam in Thai language. Phytochemical studies on *Kaempferia parviflora* revealed that rhizomes contain volatile oil (Wongsinkongman *et al*.,2003), chalcones (Herunsalee, 1987), phenolic glycosides (Azuma *et al*., 2008) and many flavonoids (Jaipetch *et al.*,1983). The *K. parviflora* has antifungal, antiplasmodial, antimycobacterial (Yenjaj *et al*., 2004), anti HIV-1 protease (Sookkongwaree *et al.,*2006), anti-allergic (Tewtrakool *et al*., 2008) and anti-gastric ulcers (Rujjanawate *et al*., 2005) activity. In Thai traditional medicine this plant is used as a health promoting tonic, body pains relieving substance and gastrointestinal disorder and curing male impotence (Trisomboon 2007).

Aforesaid plant species is an important treasure of India in general and North East India in particular. These plants are being depleted from natural habitat due to their medicinal importance and place its name under RET species (http://black turmeric.webs.com). Only one earlier worker (Mongkolchaipak *et al*., 2006) tried *in vitro* regeneration of this plant using BAP only.So the plant species with huge number of medicinal importance which is also on the verge

of being extinction was selected. In present experiment kinetin, BAP, NAA and IAA were used to observe the effect of these growth regulators on shoot and root regeneration.

3.1 Facts about Selected Plants

3.1.1 *Cyathea gigantea* Wall Ex. Hook.

Cyathea is a genus of tree ferns. The genus name *Cyathea* is derived from Greek Kyatheion, meaning "little cup" refers to the cup shaped sori on the underside of the fronds. The genus *Cyathea* contains more than 470 species. *Cyathea gigantea* (Wall. ex. Hook.) is a tree fern. The Cyatheaceae is the scaly tree fern family and includes the World's tallest tree fern, which reach heights up to 20m (Braggins and Large, 2004).Fresh rhizome of *Cyathea gigantea* used traditionally to treat white discharges, for this fresh rhizome mixed with black pepper and grind the mixture which was taken with milk for one week in twice a day (Rout *et al*., 2009).

Systematic Position of *Cyathea gigantea* Wall Ex. Hook.

Kingdom : Plantae

Division : Pteridophyta

Class : Pteridopsida

Order : Cyatheales

Family : Cyatheaceae

Genus : *Cyathea*

Specific epithet : *gigantea*

Morphological characteristics of *Cyathea gigantea* Wall Ex. Hook.

1. They are terrestrial fern usually with a single tall stem.
2. The trunk of this species is erect and as tall as 5m or more.
3. Fronds are bi or tri pinnate and 2-3m long.
4. The rachis is long, black in colour.
5. The scales are dark brown and glossy.Sori are round.

Fig 2 : *Cyathea gigantea* Wall. Ex. Hook in natural habitat

Fig. 3. ***Cyathea gigantea*** **Wall Ex. Hook.**

3.1.2 *Dioscorea alata* L.

Dioscorea alata L. a slender creeping vine reaching a length of several meters. A glabrous twinner, with a peculiar 4 winged stem. Leaves are simple, opposite, ovate, cordate and acuminate. Flowers are unisexual and fruits capsules. Plant has many aerial tubers that also aid in reproduction. Underground tubers are large many branched dark brown and fleshy. It is edible and used as a staple food.

Systematic position of *Dioscorea alata* L.

Kingdom : Plantae

Division : Magnoliophyta

Class : Liliopsida

Order : Dioscoreales

Family : Dioscoreaceae

Genus : *Dioscorea*

Specific epithet : *alata*

Morphological characteristics of *Dioscorea alata* L.

1. *Dioscorea alata* is a herbaceous twinning plant.
2. Stem 10m to more in length.
3. Internode square in cross section with red to purple tinged.
4. Aerial tubers formed in leaf axils.
5. Tubers are elongate to 10cm x 3cm with rough surface.
6. Leaves longs, opposite, blades 20cm or more long, narrowly heart shaped.
7. Flowers small, male and female flower arising from leaf axils on separate plant.

Fig.4. ***Dioscorea alata*** **L. in natural habitat**

Fig 5. ***Dioscorea alata*** **L.**

3.1.3 *Arundina graminifolia* (D. Don) Hochr.

Arundina comes from Latin word arundo which means reed and *graminifolia* means grass like leaves. *Arundina graminifolia* (D. Don) Hochr. is commonly known as bamboo orchid. Bamboo orchid is a terrestrial perennial orchid with erect stem, forming into large clumps growing to a height between 70cm to 2m. The leaves are long and narrow, with a length of 9cm- 19cm and a width of 0.8 to 1.5. The apex is acuminate. The flowers are oval-shaped and like all orchid flowers, have an outer whorl consist of 3 sepals and an inner whorl with 3 petals. The top sepal is narrow or smaller than the 2 side petals. Flowers are purple red or white tepals (petals and petals like sepals), magenta edged labellum (lip) with a yellow center. The short lived, scented flowers last for about 3 days many flowers bloom at a time. Seed capsules are 6-ribbed when it split it looks like a bird cage and release minute dust like seed in air.

The orchid blooms in summer and autumn, showing rather open clusters of open terminal flowers. They bloom in succession on the terminal racemes, which are 7cm-16cm long. These flowers are 5cm- 8cm in diameter. The occasional fertilized seed pods contain minute powdery seeds.

The genus considered to possess activities of detoxification, antiarthritis and abirritation. It is also used as antidote and demulcent. Stilbenoids are the major components in this plant (Majumder and Ghosal, 1994) and also triterpenes (Wan *et al.,* 1971).

Systematic position of *Arundina graminifolia* (D. Don) Hochr.

Kingdom : Plantae

Division : Magnoliophyta

Class : Liliopsida

Order : Orchidales

Family : Orchidaceae

Genus : *Arundina*

Specific epithet : *graminifolia*

Morphological characteristics of *Arundina graminifolia* (D. Don) Hochr.

1. Bamboo orchid is a terrestrial perennial orchid with erect stem.
2. The stem is solid and 3m in length.
3. The leaves are long and narrow with a length 9cm- 19cm and a width of 0.8- 1.5 cm.
4. Flowers occur in cluster.
5. Flowers have 3 sepals which form outer whorl and 3 petals which form inner whorl.
6. Flowers are purple red in colour.

Fig. 6. ***Arundina graminifolia*** **(D. Don)Hochr.**

3.1.4 *Kaempferia parviflora* Wall. ex.Baker

Zingiberous plants are generally herbs, often large, with a pseudostem. Leaves are generally radial and cauline, membranous. Sheaths are large, lamina with a strong central nerve and close secondary nerve. Hermaphrodite flowers are present. This species hold their leaves more upright than most. They tend to arch out from the centre of the clump. The species name *parviflora* refers to the small flowers which are normally hidden below the foliage.

Systematic position of *Kaemferia parviflora* Wall ex. Baker

Kingdom : Plantae

Division : Magnoliophyta

Class : Liliopsida

Order : Zingiberales

Family : Zingiberaceae

Genus : *Kaempferia*

Specific epithet : *parviflora*

Morphological characteristics of *Kaempferia parviflora* Wall ex. Baker.

1. *Kaempferia parviflora* is a small, stemless rhizomatous herb up to 20cm tall.
2. Leaves one to several, 8-10 cm long, obovate to elliptic.
3. Flowers few in a sessile central tuft.
4. Corolla white in colour, white with purple blotch at the middle.
5. Rhizome small and deep purple coloured.

Fig.7. *Kaempferia parviflora* Wall ex. Baker. in natural habitat