CHAPTER - II

MICROPROPAGATION

2.1. INTRODUCTION

Micropropagation is the true to type propagation of selected genotype using in vitro culture techniques. This technique provides a rapid reliable system for production of large number of genetically uniform and disease free plantlets. Through this technology (Musa sapientum cv.) from a single shoot tip or axillary bud, a large quantity of uniform and disease free plants with good genetic potential can be produced within a short time (Vuylsteke and Langhe, 1985; Wong, 1986 and Akbar and Roy, 2006). Tissue culture has become crucial in the ex situ conservation of rare and endangered plant species. Micropropagation technique has been successfully undertaken for many plants and was considered the only viable method for Symonanthus bancroftii due to its extreme rarity, vulnerability and urgency for off-site propagation (Panaia et al., 2000).

Micropropagation procedure for the multiplication of desired or selected genotypes of Panicum virgatum has been reported by Alexandrova et al. (1996). Shoot tip culture was considered an important system for micropropagation. Micropropagation was successfully utilized by meristematic shoot segments excised from mature embryo derived seedlings of barley (Sharma et al., 2004a). Simple, genotype independent and efficient method for plant regeneration using shoot tip explants of Pearl millet (Mythili et al., 2001) and Sorghum (Baskaran and Jayabalan, 2005) has been reported.

To start a breeding program applying biotechnological approaches the establishment of in vitro micropropagation technique is required as the first step. This fact converts the tissue culture into another relevant tool of the plant breeding process. In this sense, the micropropagation of selected or improved plants allows the permanent propagation of selected traits within a specific genotype (Horn, 2002 and Alderete et al., 2006).

During the last thirty years, micropropagation and other in vitro techniques have become more widely used in commercial horticulture and agriculture for the mass propagation of crop plants and for conservation of genetic resources, particularly with
those crops which are vegetatively propagated or have recalcitrant seeds which cannot be stored under conventional seed bank conditions (George and Sherrington, 1984; Dodds, 1991 and George, 1993). Likewise, in vitro culture is being used in an increasing number of botanic gardens for the propagation and conservation of wild plant species (Fay, 1992).

In vitro micropropagation methods have been reported for the medicinal plant species Kaempferia galanga (Shirin et al., 2000), Centella asiatica (Tiwari et al., 2000), Piper longum (Soniya and Das, 2002a), Sophora flavescens (Zhao et al., 2003), Erycoma longifolia (Hussein et al., 2005), Curcuma zedoaria (Loc et al., 2005) and Wattakaka volubilis (Chakradhar and Pullaiah, 2006), endemic plant species Centaurea paui (Cuenca et al., 1999) and Heliotropium keralense (Sebastian et al., 2006a) and endangered plant like Symonanthus bancroftii (Panaia et al., 2000).

Nodal segments from inflorescence stems have proved successful as starting material for micropropagation of field grown plants for different rare or threatened species growing in the vegetative phase as rosettes of leaves such as Limonium cavanillesii (Marco and Ibanez, 1998). Virus free plant can be obtained from five variable methods such as heat treatment followed by meristem culture. Adventitious shoot formation followed by meristem culture and grafting of meristem on virus free rootstocks or micrografting (Huda and Sikdar, 2006 and Pierik, 1997) were also practiced.

Micropropagation using nodal explants has been reported in many plants Tectona grandis (Tiwari et al., 2002), Ocimum basilicum (Sudhakaran and Sivasankari, 2003), Cinnamomum camphora (Babu et al., 2003) and Solanum trilobatum (Jawahar et al., 2004), shoot tip explants in Ranunculus asiaticus (Beruto and Debergh, 2004), Gloriosa superba (Hassan and Roy, 2005) and Thuja occidentalis (Kabir et al., 2006), axillary buds in Paeonia suffruticosa (Beruto et al., 2004) and Rubus pubescens (Debnath, 2004) and rhizome explants in Acorus calamus (Gavhane and Mukundan, 2006).

Recently, micropropagation protocol have been developed for many important medicinal plants such as Drosera indica (Jayaram and Prasad, 2007), Panax japonicus (You et al., 2007), Tylophora indica (Faisal et al., 2007), Ulmus parvifolia (Thakur and Karnosky, 2007), Crataeva nurvula (Walia et al., 2007), Eclipta alba (Ray and Bhattacharya, 2008), Basilicum polystachyon (Amutha et al., 2008), Huernia hystrix (Amoo et al., 2009), Jatropha curcas (Purkayastha et al., 2010) and Centaurea ultreiae (Mallon et al., 2010).
Hence, present study was undertaken to standardize an effective and reliable in vitro method for shoot proliferation and micropropagation of this species. This study deals with the successful micropropagation system for production of plants in large-scale from both in vitro and in vivo derived (node, apical bud and axillary bud) explants of Plectranthus barbatus.

2.2. REVIEW OF LITERATURE

Relevant literature pertaining to the present investigation is presented here. Simola (1978) reported micropropagation of Drosera rotundifolia by culture of young seedling explant. Anthony (1992) and Kukulczanka and Czastka (1988) described in vitro propagation of Drosera species by leaf and axillary bud culture and Jang and Park (1999) reported mass propagation of Drosera rotundifolia through shoot and shoot tip culture. Traditionally, propagation of tuberous sundews was carried out (Kim and Jang, 2004) with seeds leaf cutting and secondary tubes.

Micropropagation of Alnus nepalensis was achieved using axillary bud explant cultured on MS medium supplemented with KN (0.5mg/l) and BAP (0.5mg/l). In vitro shoots were transferred to culture tubes for root regeneration on ¼ strength of MS medium fortified with 1.0mg/l IBA. The rooted plants were successfully transferred to pots containing a mixture of soil and sand (Kaur et al., 1993).

Flower buds of peach palm plants (Bactris gasipaes) were micropropagated by Almeida and Kerbauy (1996). The best results were obtained with 0.8mg/l BA + 2.4mg/l NAA. Rooting was induced in a medium devoid of plant growth regulators. Elongated flower buds of B. gasipaes were cultured in the presence only of 2.4mg/l NAA, only roots were formed at their basal portion.

Induction of multiple shoots using cotyledonary nodes and shoot tips of Macrotyloma uniflorum was described by Mohamed et al. (1999). The highest rate of shoot proliferation came from MS medium added with BAP 1.5mg/l. The highest frequency of shoot proliferation and elongation was obtained on MS medium containing BAP (1.5mg/l) along with AdS (1.0mg/l) and GA3 (0.5mg/l), then the rooted shoots gave rise to the medium containing 1.75mg/l IBA. Sivaprakash et al. (1994) reported that BAP was more efficient in producing shoots in Cajanus cajan.
Tanuwidjaja et al. (1998) and later Soniya and Das (2002b) carried out the best shoot proliferation was obtained from the distal half of stem explants in 8.8μM BA. High frequencies of rooting were obtained on half MS + 2.46μM IBA for two weeks. Tiwari et al. (2002) reported that the propagation of teak via., cuttings has several limitations and only provided a few propagules from selected individuals. Poor explant response and rapid explant browning were major hurdles to successful establishment of teak in vitro.

A micropropagation protocol for Pothomorphe umbellata was carried out by Pereira et al. (2000) using leaf segments cultured on ¼ strength MS medium supplemented with 0.5mg/l BA, 0.1mg/l GA3 added with 10g/l sucrose. Rooting was achieved using MS medium devoid of growth regulators. An anatomical study confirmed shoot regeneration via., direct organogenesis.

Lisowska and Wysokinska (2000) worked on Catalpa ovata and regenerated in vitro from shoot tip explants on SH (Schenk and Hildebrandt, 1972) or WP (Lloyd and McCown, 1980) basal media supplemented with BA (4.4μM) alone or in combination with IAA (0.6μM) and nodal explants on same media supplemented with BA (2.2μM) alone or in combination with IAA (0.6μM). WP medium was chosen because it has been used widely in woody plant cultures; SH medium has been used successfully for the micropropagation of Catalpa bignonioides (Wysokinska and Swiatek, 1989). The synergistic effect of cytokinin and auxin has been reported for Albizia lebbeck (Upadhyaya and Chandra, 1983).

Fracaro and Echeverrigaray (2001) were micropropagated through axillary buds of Cunila galioides Benth. The highest rate of shoot proliferation were obtained using MS medium supplemented with 8.8μM of BA. The best conditions for rooting were MS medium + 0.5 to 2.5μM of IBA.

Sivasubramanian et al. (2002) standardized a method for the micropropagation of Plectranthus vetiveroides. Axillary shoots from nodal explants of adult plants could be stimulated to undergo multiple shoot formation (12.8 ± 0.25) on MS medium containing 4.44μM BA. In vitro shoots were rooted on half strength MS medium supplemented with IBA (7.38μM).
Dode et al. (2003) described the procedure for micropropagation of *Ocimum basilicum* using cotyledonary leaf from *in vitro* geminated plants. Cotyledon explants were put in MS (Murashige and Skoog, 1962) medium with 0.2mg/l NAA in combination with 0 – 5.0mg/l BAP. The highest efficiency of shoot formation after 45 days occurred in the medium containing 5.0mg/l BAP and 0.2mg/l NAA. The presence of NAA inhibited root formation, when combined with different concentrations of cytokinin.

Wala and Jasrai (2003) established multiple shoots from meristem tip of *Curculigo orchioides* cultured on MS medium supplemented with BA (2.21µM). The shoots were rooted on half strength of MS basal medium supplemented with NAA (0.53µM).

Sudhakaran and Sivasankari (2003) micropropagated *Ocimum basilicum* using apical bud and nodal explants which were excised from the plant and inoculated on MS medium fortified with two cytokinin BAP (2.0mg/l) and KN (1.5mg/l) and achieved optimum (100%) response in shootlets formation. Ahuja et al. (1982) obtained multiple shoots from *O. americanum* and *O. gratissimum* on the media containing 5.0mg/l BAP and 0.5mg/l IAA. Pattnaik and Chand (1996) reported that *O. americanum* and *O. sanctum* with BA in the range of 0.25 - 2.0mg/l stimulated bud break with 88% bud sprouting. Multiple shoot induction and bud break declined with an increase in the concentration of BA above the optimal levels. The stimulatory effect of BA on bud break and multiple shoot formation was reported in *Mentha* species (Rech and Peris, 1986). Sudhakaran and Sivasankari (2003) found in *O. basilicum* NAA (3.0mg/l) was more effective for root formation whereas in *O. sanctum* rooting was reported on 1.0mg/l NAA and in *O. americanum* on 1.0mg/l IBA (Pattnaik and Chand 1996).

Haw and Keng (2003) micropropagated using axillary bud explants of *Spilanthes acmella* on MS medium supplemented with 2.0mg/l BA. MS medium containing 0.5mg/l BA was sufficient for the proliferation of rooted multiple shoots. Eellarova and Kimakova (1999) reported that BA was found to be most effective for induction of multiple shoot formation when the concentration was more than 1.0mg/l for *Hypericum perforatum*. Su et al. (2000) established that MS medium added with low concentration of BA produced normal shoots and roots for *Typhonium flagelliforme*. 
Shanthi and Xavier (2003) standardized a protocol for micropropagation of *Enicostemma littorale* through *in vitro* culture and rapid multiplication. Multiple shoots (87 shoots/explant) were harvested on MS medium fortified with BA 2.5 mg/l. *In vitro* derived shoots were rooted on MS medium containing NAA (0.2 mg/l) and IBA (0.1 mg/l). The well rooted plantlets were hardened.

A micropropagation system based on the young stem node segments of *Sophora flavescens* was established by Zhao *et al.* (2003). MS medium supplemented with 8.88 μM BA + 2.69 μM NAA and that with only 5.37 μM NAA were found the best in promoting proliferation of shoots and roots, respectively. Beena *et al.* (2003) achieved a protocol for rapid *in vitro* propagation of *Ceropegia candelabrum* through axillary bud multiplication. MS medium 8.87 μM BA and 2.46 μM IBA was best suited for axillary bud proliferation in nodal segments. Shoots developed were rooted best on half strength MS with 0.49 μM IBA.

Reeth and Shivamurthy (2004) achieved *in vitro* micropropagation of *Nyctanthes arbor-tristis* through nodal explant culture on MS medium containing BAP (1.5 mg/l). Regenerated shoots elongated in MS medium containing BAP (1.5 mg/l) and rooted on half strength MS liquid medium with NAA (0.5 mg/l) and IBA (1.0 mg/l). *In vitro* plantlets were hardened using Vermiculite: Soil (1:1). Poornima and Shivamurthy (2004) attempted the clonal propagation of *Tristellateia australis* using nodal explants. Shoots regenerated from axillary meristem on MS medium supplemented with BAP (1.0 mg/l) and NAA (2.0 mg/l). The increased shoots were achieved on medium with CM (20%) and NAA (1.0 mg/l). The shoots were rooted on MS medium with NAA (2.0 mg/l).

Prasad *et al.* (2004) developed a protocol for micropropagation of *Cryptolepis buchanari* by using shoot tip, cotyledonary node and nodal explants. The maximum number of shoots (12.5 shoots/explant) were produced on MS medium fortified with BAP 2.0 mg/l, KN 0.1 mg/l, NAA 0.05 mg/l and GA3 0.5 mg/l with 60% response. Individual shoots were rooted on MS medium supplemented with IBA 1.0 mg/l. *In vitro* raised plantlets were acclimatized successfully to pots.

Shrottri and Mukundan (2004) cultured nodal segments of *Rubia cordifolia* on MS medium enriched with TDZ 4.54 μM, that induced 10 shoots 1.33 cm length in 80% of the cultured explants within 8 weeks of culture. Node and shoot tip explants excised from
in vitro grown shoots when subcultured in 2.27μM TDZ containing medium produced 15.6 shoots per node and 16.8 shoots per shoot tip explants in 6 weeks. In vitro grown shoots formed roots in MS medium supplemented with 9.8μM IBA. Evenor and Reuveni (2004) described a method for large scale propagation of Achillea filipendulina through meristem cultures. The best shoot proliferation was found to be in MS medium supplemented with 3% sucrose and 1.0mg/l IAA + 2.0mg/l BA under 16 hrs of cool fluorescent light.

The highest rate of multiple shoot proliferation was observed on MS fortified with BAP (8.88μM/l) followed by KN 9.28μM/l respectively both node and shoot tip explants of Solanum trilobatum. The well developed shoots were rooted on MS medium supplemented with IBA 9.48μM/l (Jawahar et al., 2004). Micropropagation has proved to be an alternative for the multiplication of selected genotypes and chemo types of several medicinal and aromatic plants (Fracaro and Echeverrigaray, 2001). Vegetative propagation of tree species remains problematic, that is why micropropagation was investigated using axillary buds, as well as the possibility for regeneration via., adventitious buds (Beruto et al., 2004).

Highly efficient short term in vitro regeneration system was developed by Sharma et al. (2004b) on Hordeum vulgare. Meristematic shoot segments excised from mature embryo derived seedlings and subsequently cultured on MS basal medium containing 2.0mg/l piclorum and 3.0mg/l thidiazuron differentiated up to ten multiple shoots after 3 to 4 weeks. Clumps of multiple shoots were further multiplied on proliferation maintenance medium containing 2.0mg/l piclorum and 2.5mg/l TDZ. The differentiated small shoots and shoot buds gave rise to normal shoots on medium with 0.1mg/l piclorum and 1.0mg/l TDZ. Shoots were rooted on basal MS medium with 0.5mg/l or 1.0mg/l IBA the plantlets were transferred to the soil and showed normal growth.

Debnath (2004) standardized a protocol for micropropagation of dwarf raspberry through induction of shoot proliferation and rooting in vitro. Cultures were initiated from shoot tip and nodal explants on half MS medium containing 8.9μM BA and 0.98μM IBA. Zeatin was more effective than BA. Shoots growing for more than 10 weeks on medium that contained 9.1μM zeatin occasionally produced adventitious shoot masses. Shoots
were rooted *in vitro* in the same medium used for shoot proliferation without any growth regulators. A protocol was described by Sharma *et al.* (2004a) for rapid and large-scale *in vitro* propagation of *Anethum graveolens* using axillary shoot segments. The synergistic combination of 0.5mg/l BAP and 0.1mg/l IBA induced 100% shoot formation. The rooting of shoots was achieved on a medium with 1.0mg/l IBA and 0.5mg/l KN.

Baksha *et al.* (2005) effected micropropagation of *Aloe barbadensis* through shoot tip explants cultured on MS medium fortified with BAP (2.0mg/l) and NAA (0.5mg/l). About 95% rooted plants were obtained from microshoots cultured on half strength MS medium containing NAA (0.5mg/l). *Crinum macowanii*, the endangered level of BAP had a stimulating effect on the total number of regenerated plantlets (Slabbert *et al.* 1993). Liao *et al.* (2004) reported that a combination of BAP (2.0mg/l), NAA (0.3mg/l) and PVP (0.6mg/l) enhanced the multiple shoot proliferation from shoot tip explants of *Aloe barbadensis*. In contrast, best multiplication medium reported by Chaudhuri and Mukundan (2001) was supplemented with 10mg/l BAP + 100mg/l AdS with 0.1mg/l IBA. This might be due to genotypic variation of explants reinforced by the cultural and environmental conditions.

An efficient plant regeneration system was established by He *et al.* (2005) via., organogenesis from shoot tip explants of *Aquilaria agallocha*. Shoots regenerated many buds on MS medium supplemented with 1.3µM BA then the shoot buds were elongated on same media with 1.3µM BA + 0.5µM NAA. Plantlets were rooted half strength MS medium after being immersed in 5.0µM NAA.

Regeneration of multiple shoots from different *in vitro* grown explants viz., shoot tip, nodal segments and cotyledonal node of *Peltophorum pterocarpum*. Cotyledonal node explants gave best shoot multiplication compared to other explants on MS media containing 2.0mg/l KN + 0.5mg/l NAA. In some cases BAP also showed better results. The regenerated shoots were transferred to MS media having IBA for adventitious root initiation (Uddin *et al.*, 2005). Cotyledonal node of cotton produced maximum number of shoots (3.43 shoots/explant) when cultured on MS medium supplemented with 0.25mg/l KN and the highest percentage (93.3%) of root development and root length (5.85 cm) was obtained when shoots were cultured on MS medium supplemented with 0.5mg/l NAA.
and 0.1mg/l KN. Hemphil et al. (1998) observed the best development of shoots from *Gossypium hirsutum* on MS medium containing 0.3µM BA. Multiple shoots elongated within the same medium. Agarwal et al. (1997) obtained multiple shoots by culturing cotyledonary nodes devoid of apical meristem in MS basal medium supplemented with 2.5mg/l each of BAP and KN. Saeed et al. (1997) achieved best development of roots on medium containing 2.68mM NAA and 0.46mM KN. Effect of the type of cytokinin used for *in vitro* shoot proliferation on the subsequent rooting of shoots was studied by Bennett et al. (1994). They found, that shoots from the multiplication medium containing KN produced more roots and remained healthy for a longer period on the rooting medium as compared to shoots taken from multiplication medium containing BAP.

Micropropagation protocol was developed using nodal shoot segments of mature plant of *Maerua oblongifolia* (Rathore et al., 2005). Nodal segments were differentiated into multiple shoots on MS medium + 0.1mg/l IAA + 5.0mg/l BAP + additives (25.0mg/l each of adenine sulphate, L-arginine and citric acid and 50.0mg/l ascorbic acid). Shoots were further multiplied by repeated transfer of subculture on MS medium + 0.1mg/l IAA + 1.0mg/l or 0.5mg/l BAP + additives. Micropropagated shoots rooted on half MS basal medium + 0.5mg/l IBA. Rooted plantlets were hardened in a greenhouse.

Velayutham et al. (2005) reported that *in vitro* multiple shoot formation were observed from nodal explants of *Plumbago zeylanica* on MS medium supplemented with treatment of cytokinin. Maximum number of shoots was achieved in micropropagation resulting from nodal explants on MS basal medium fortified with 10.0µM BAP and 3% (w/v) sucrose. Rooting of the differentiated shoots was achieved in media having 3.0µM NAA with 3% sucrose within 20 days of culture.

Multiple shoots were induced from shoot tip explant of *in vitro* grown seedlings of *Arachis hypogaea* on MS medium supplemented with 2.0mg/l BAP, 0.2mg/l AdS and 0.2mg/l GA3. The plantlets were transferred to rooting media containing 2.0mg/l NAA. Rooted plantlets were successfully transferred to the field after hardening (Bhanumathi et al., 2005).

An efficient method for micropropagation of *Daucus carota* was investigated by Yazhisai et al. (2005). Maximum number of multiple shoots were obtained when culturing
shoot tips on MS medium fortified with BAP (1.0mg/l) + KN (1.0mg/l) + Glutamine (10mg/l) and maximum number were obtained from nodal explants cultured on MS medium fortified with BAP (2.0mg/l) + KN (0.5mg/l) and Glutamine (5.0mg/l). The shoots elongated rapidly on KN (0.5mg/l) + GA3 (0.5mg/l) + BAP (1.5mg/l). IAA at 0.25mg/l was found to be the best for rooting of shoots.

Loc et al. (2005) reported an efficient micropropagation system for Curcuma zedoaria Roscoe using rhizome sprout cultures. Shoots were induced from rhizomes on basal MS medium containing 20g/l sucrose and 5g/l agar, supplemented with 20% (v/v) coconut water and BA (0.5 - 5.0mg/l). The highest shoot multiplication was obtained on MS medium with 20% (v/v) coconut water, 3.0mg/l BA and 0.5mg/l IBA. Well developed shoots were rooted on MS medium containing 20g/l sucrose and 8g/l agar, supplemented with 20% (v/v) coconut water and 2.0mg/l NAA. Rooted plants were established in pots after hardening.

High frequency shoot proliferation was achieved by using apical and axillary buds of Gloriosa superba on MS basal medium fortified with 1.5mg/l BA + 0.5mg/l NAA. Addition of 15% (v/v) coconut water and 20g/l activated charcoal increased the number of shoots up to 15 per culture. In vitro shoots were rooted on MS medium with 1.0mg/l IBA + 0.5mg/l IAA. Plantlets could be hardened and grew well after transfer to greenhouse (Hassan and Roy, 2005).

In vitro micropropagation (Hussein et al., 2005) was carried out by using shoot tip explants of Eurycoma longifolia. The highest number of shoots was obtained on basal MS medium supplemented with 5.0mg/l KN. Shoots were rooted on MS medium supplemented with 0.5mg/l IBA. Plantlets did not exhibit morphological differences from parent plants after two months of transplantation to soil.

Rapid micropropagation protocol was developed for multiplication of plantlets through direct regeneration from three locally grown chickpea (Cicer arietinum L.). The highest percentage of multiple shoots was achieved from cotyledonary node explants on MS and B5 containing 4 × MS microsalts, 3.0mg/l BAP and 0.04mg/l NAA in all the three varieties of chickpea. Rooting efficiency was seen on MS containing 0.2mg/l BAP and 0.5mg/l NAA in all the three varieties. Micrografting technique has been found to be a good alternative to in vitro rooting (Sarker et al., 2005).
Ran and Simpson (2005) have developed a protocol for *in vitro* propagation of the genus *Clivia*. Multiplication from initiations and *in vitro* seedlings was the best, when the explants were cut longitudinally through meristem and placed on MS + 44μM BA. Plantlets were transferred on to hormone free MS medium with charcoal for rooting. *In vitro* orchid micropropagation protocol for *Cymbidium aloifolium* and *Rhyncostylis retusa* was achieved by Bordoloi (2005). The results showed that the percentage of seed germination of *R. retusa* and *C. aloifolium* was highest (99%) in cultures grown on Knudson (1946) (KnC) medium containing IBA (1.0μg/ml) and BA (2.5μg/ml) and PLB formation and shoot multiplication on KnC medium with IAA (1.0μg/ml) and BA (5.0μg/ml). Highest rooting success was obtained in half strength medium of KnC or MS with 2.0μg/ml IBA.

Hussein *et al.* (2005) reported that the most common method of propagating *Eurycoma longifolia* is through seeds. However, being recalcitrant they have a low percentage of germination and it takes a long time to germinate due to extremely immature state of zygotic embryo at the time of dispersal. The existence of tissue culture technology can play an important role in this regard, with the added advantage of maintaining disease free plants (Aly *et al*., 2002).

A successful *in vitro* micropropagation protocol was achieved by Kumar *et al.* (2003) using nodal explants of *Tinospora cordifolia* on MS medium supplemented with NAA(0.06mg/l) and BA (2.0mg/l), whereas BA induced only multiple shoots, NAA along with BA induced both shoots and roots. Regenerating cultures were subjected to different concentrations of Copper sulphate (25 - 125μM) and Zinc sulphate (25 - 75μM) to monitor morphogenic events.

A simple micropropagation method was done by Rajeswari and Paliwal (2006) in *Pterocarpus santalinus* L. The maximum shoot multiplication was obtained from cotyledonary node explants on MS medium containing 2.5μM of BAP. Best results (82.5% rooting and 100% survival) were observed in *ex vitro* rooting. Dipping the basal end of shoots in 5.0μM IAA solution for 25 days induced maximum rooting. The micropropagated plants were successfully transferred to field condition with 80% survival rate.
An efficient, rapid and large-scale propagation of the elite exotic, fruit yielding, medicinal plant of *Cyphomandra betacea* was evolved through *in vitro* axillary bud multiplication. Most effective shoots were obtained on MS medium supplemented with 5.0mg/l BAP. By repeated subculturing on the primary inductive media, a high frequency multiplication rate was established. Efficient rooting was achieved with 1.5mg/l IBA. Rooted microshoots were passed through acclimatization and established in soil (Chakraborty and Ray, 2006). Sharma and Vimaladevi (2006) established an efficient protocol for *in vitro* multiplication and conservation of *Peristrophe bicalyculata* Nees, a lesser known medicinal plant. Single node explants cultured on MS medium supplemented with 0.5mg/l BA and 0.1mg/l IAA produced six shoots within 8 weeks of culture. Rooting was readily achieved by transfer of the shoots on half strength MS basal medium. Plantlets were acclimatized and transferred to soil.

An efficient and reproducible protocol (Sebastian *et al.*, 2006a) was established for rapid micropropagation of *Heliotropium keralense* through shoot tip culture. Murashige and Skoog’s (1962) medium supplemented with BA (3.0mg/l) and KN (3.0mg/l) was the most effective combination of the induction of multiple shoots. MS medium supplemented with IBA (0.5mg/l) was suitable for rooting of shoots. Plantlets with well developed roots were hardened under controlled condition and eventually established in the field. Morphologically there was no detectable variation between *in vitro* raised and naturally grown plants.

Sebastianraj *et al.* (2006) established *in vitro* seed germination and plantlet development of *Coelogyne mossiae*. Seed germination was higher in Knudson C medium (95%) and lower in half strength MS medium (65%). After 5 months, when plantlets had produced 2 - 3 leaves, they were transferred to hormone supplemented half strength MS medium and Knudson C medium. Half MS medium supplemented with BAP (0.2mg/l) proved better for multiplication of protocorm and healthy shoot induction. Plantlets were transferred to auxin supplemented half strength MS medium for root induction. High frequency of roots was observed in the medium fortified with auxin (0.1mg/l). Then rooted plantlets were transferred to a paper cup for successful hardening.

Alderete *et al.* (2006) studied the *in vitro* behavior of *Mecardonia tenella* and to establish a routine protocol for its micropropagation. *In vitro* establishment of *M. tenella*
nodal segments were disinfected by standard methods using ethanol/sodium hypochloride and cultured on hormone free medium supplemented with a mixture of antibiotics and antifungal agents. Nodal segments were cultured on MS medium supplemented with antibiotics/antifungal and combination of BAP and NAA. The best results were obtained in the treatment containing BAP (0.25mg/l and 0.5mg/l) with multiplication rate of 32 shoots per explant. The regenerated shoots rooted spontaneously.

A reproducible protocol has been developed for in vitro micropropagation of *Thuja occidentalis* through apical shoot culture. Hormone free MS medium 100% explants produced shoots. The average number of shoot length of 4.5 cm was recorded in this medium. Shoots were rooted on half MS medium supplemented with IBA (1.0mg/l). No morphological variants were observed during the passage of in vitro culture (Kabir et al., 2006).

An efficient protocol has been developed for in vitro plant production through apical meristem culture of *Momordica charantia* L. The growth of meristem was observed on semisolid MS medium supplemented with KN (0.05mg/l) + GA₃ (0.1mg/l) and they were transferred to MS medium supplemented with BA, KN, IBA and IAA singly or in combination for shoot elongation and root induction. Good shoot initiation with elongation was obtained in MS medium supplemented with BA (1.0mg/l) + IBA (0.1mg/l) + GA₃ (0.3mg/l) and good rooting was observed when IBA (0.5mg/l) and NAA (0.1mg/l) were used in MS semisolid medium. Old plantlets were successfully planted in soil through gradual acclimatisation (Huda and Sikdar, 2006).

In vitro flowering and an efficient micropropagation protocol was developed for *Vitex negundo* using nodal segments of mature plant (Vadawale et al., 2006). Fully functional flowers were observed on MS medium fortified with 4.4μM BAP and 0.53μM NAA, with an average of five shoots/node and was the best for axillary bud proliferation. Full MS medium containing 3.69μM IBA exhibited the best in vitro rooting and 95% of rooted plantlets survived, when transferred to green house.

Chakradhar and Pullaiah (2006) standardized micropropagation protocol for multiplication of shoots from nodal explants of *Wattakaka volubilis* (L.f.) Stapf. Nodal explants of in vivo and in vitro seedlings were cultured on MS medium fortified with
cytokinins BA (0.5 - 5.0mg/l), KN (0.5 - 10mg/l), TDZ (0.05 - 1.0mg/l) either singly or in combination with NAA (0.1mg/l). Maximum number of shoots (14.1) with 80% regeneration frequency was obtained from nodal explants of seedlings cultured on KN (5.0mg/l) + NAA (0.1mg/l). The differentiated shoots from both could be rooted with 85% frequency on half strength MS medium (1% sucrose) with 0.6% agar + IBA (1.0mg/l) + KN (0.2mg/l). Rooted shoots were transferred to vermiculite with soil (3:1).

Bay et al. (2007) standardized an in vitro propagation of Hornstedtia reticulata through meristem explants. The sterilized seeds were sown on Gamborg (B5) medium. The meristems of 12 weeks old seedlings were used to induce multiple shoots in Gamborg (B5) media incorporated BAP alone and in combination with NAA. Frequency of shoot proliferation was highest at 3.0 mg/l BAP alone, with the average number of shoots was 9.67 after three subcultures.

An efficient protocol was described for the rapid in vitro multiplication of an endangered medicinal plant, Tylophora indica via., enhanced axillary bud proliferation from nodal explants collected from young shoots of a two year old plant (Faisal et al., 2007). The highest number (8.6 ± 0.71) of shoots and the maximum average shoot length (5.2 ± 0.31cm) were recorded on MS medium supplemented with 2.5µM BA, 0.5µM NAA and 100mg/l AA at pH 5.8. Rooting was best achieved on half strength MS medium augmented with 0.5µM IBA.

Thakur and Karnosky (2007) achieved a simple and efficient protocol system for micropropagation, germplasm conservation and distribution using Ulmus parvifolia. Newly flushed nodal segments were used as explants. WPM with 0.5mg/l BA was found to be the best medium for meristematic shoot development and WPM supplemented with 2.0mg/l 4-CPPU and 0.5mg/l TDZ was best for meristematic nodule formation. Rhizogenesis of regenerants and micro-cuttings was best achieved on WPM with 1.0mg/l NAA and 2% sucrose.

An efficient, cyclic, two-step protocol for micropropagation of medicinal tree, Crataeva nurvula has been successfully developed by Walia et al. (2007), which can be employed at a commercial scale. Nodal explants from 30 years old tree when cultured on MS medium supplemented with 2.22µM BAP produced multiple shoots, which elongated
satisfactorily on the same medium. Nodal and leaf explants from *in vitro* regenerated microshoots too developed shoots, thus making the process recurrent. Addition of casein hydrolysate significantly increased the average number of shoots per explant. Maximum number of shoots regenerated on medium supplemented with 100mg/l casein hydrolysate. Shoots could be rooted on 1/2 MS supplemented with 0.11 and 0.54µM NAA.

A novel rapid micropropagation protocol was developed by Thakur and Kumar (2007) through tissue culture of *Mussaenda erythrophylla*. Nodal stem cuttings were cultured on MS basal medium containing 2.0mg/l KN and 0.5mg/l NAA for proliferation of best multiple shoots induction. Best rooting of microshoots was observed on half strength basal MS medium supplemented with 2.0mg/l of NAA.

Micropropagation protocol was achieved by Hamirah *et al.* (2007) from shoot tips of *Boesenbergia pulchella*. Explants were cultured on Gamborg (B5) medium containing 3% (w/v) sucrose and 0.8% (w/v) Gelrite. The highest number of shoots was obtained on B5 medium supplemented with BAP at 3.0mg/l + NAA 0.1mg/l with a mean of 6.8 shoots/explant. A maximum of 11.0 shoots were produced after treatment with TDZ at 0.3mg/l, which were the highest among other treatments.

A protocol has been developed for micropropagation of two economically important bamboos. The multiple shoots were excised from mother explant and further multiplication was observed on MS medium supplemented with 3.0mg/l BAP for *Drepanostachyum falcatum* and 3.0mg/l BAP with 0.5mg/l KN for *Bambusa balcooa*. *In vitro* shoots were rooted when transferred to MS medium supplemented with IBA, NAA and IAA. The rooted plantlets were hardened, acclimatized and successfully transferred to the field (Arya *et al.*, 2008).

A protocol for micropropagation of *Tabebuia aurea* through nodal explants has been developed by Rani *et al.* (2008). MS medium fortified with 2.0mg/l BAP + 0.5mg/l KN achieved regeneration with optimum shoot length. *In vitro* shoots rooted on IAA 1.0mg/l. The effect of rooting hormones was observed for (*Terminalia chebula*) five concentrations (1000, 2000, 3000, 4000 and 5000ppm) of IBA, IAA and NAA. The maximum rooting response was observed in case of IBA 4000ppm (55%) treated cuttings followed by IBA and IAA 3000ppm (35%) treatments (Madhwal *et al.*, 2008). Direct organogenesis and *in vitro* flowering obtained in *Basilicum polystachyon*. High frequency
and maximum number of multiple shoots were obtained from shoot tip explants on MS medium supplemented with BAP (8.88µM) and KN (9.22µM). Regenerates, when transferred to rooting medium IBA (9.84µM) and IAA (11.42µM), initiated flowering along with rooting. Rooted plantlets were hardened and transferred to green house with 100% survivability (Amutha et al., 2008).

An efficient method of micropropagation for *Eclipta alba* from young nodal axils of shoot tip explants has been developed by Ray and Bhattacharya (2008). Among 3 cytokinins BAP, KN and TDZ, BAP was found to be most effective in inducing and proliferating adventitious shoots. The highest frequency of responding explants (100%) and maximum number of shoots (23.0) per explant were obtained after 60 days culture on MS medium containing 8.8µM BAP. All shoots developed roots directly from shoot bases when transferred to growth regulator-free MS medium.

Lakshmiprabha et al. (2008) described *in vitro* mass propagation of *Andrographis lineata*, through nodal explant on different media, amino acid, carbohydrates and in various concentrations of growth regulators. The highest 94.2 percentage of shoot proliferation was obtained by placing nodal explant on MS salt with B5 vitamin supplemented with 1.5mg/l 2iP in combination with 0.6mg/l KN and glutamine 20mg/l, which produced 7.8 shoots per explant. Regenerated shoots were rooted, when transferred in same medium containing 2.0mg/l IBA.

An efficient and reproducible *in vitro* protocol for large-scale multiplication of *Bambusa balcooa* has been described (Mudoi and Borthakur, 2009). Multiple shoot formation was observed from tender node when they were implanted on MS medium augmented with BAP (1.0mg/l). Continuous shoot proliferation was obtained when frequent subculture of same medium with BAP (1.0mg/l). Micro shoots rooted when transferred on MS medium supplemented with BAP (1.0mg/l) and NAA (3.0mg/l). A simple protocol for *in vitro* direct shoot multiplication was standardized by Velmurugan et al. (2009) through shoot tip explants of *Coleus forskohlii*. The maximum shoot proliferation (99%) was noticed in BAP (2.0mg/l). For best multiplication results, fortification with (0.5 mg/l) BAP produced longest shoots with great number of leaves. The best growth regulator treatment for rooting of Coleus microshoots was observed with IBA (0.2mg/l) in half MS medium.
The effect of different cytokinins on multiple shoot regeneration from shoots of Centaurea ulitreia were studied by Mallon et al. (2010). The highest multiplication rate (5.52 shoots per explant) were obtained in the medium supplemented with 4.44µM BA. Shoots were successfully rooted by dipping the basal end into a solution containing 10 M NAA for 30 sec. Genetic stability of the regenerated plants were assessed by random amplified polymorphic DNA (RAPD) analysis and flow cytometry. An efficient and reproducible in vitro plant regeneration system from shoot apices were developed by Purkayastha et al. (2010) on Jatropha curcas. The BAP 2.5µM was most effective in inducing an average of 6.2 shoots per shoot apex. Incorporation of GA3 0.5µM to basal medium was found essential for elongation of shoots. The shoots rooted efficiently on half strength MS medium. The rooted plantlets were acclimatized with more than 98% success and the plants transferred to soil.

2.3. MATERIALS AND METHODS

2.3.1. Plant Material (Source of Explants)

Plectranthus barbatus from natural habitats were collected from the Palni hills of the Western ghats. Sterilized plants were inoculated and regenerated in in vitro lab condition. Naturally growing in vivo explants of node, axillary bud and apical bud and in vitro derived node, axillary bud and apical bud explants were excised with sterile blade and collected from young plants for the present investigation.

2.3.2. Sterilization of Explants

Explants like node, axillary bud and apical bud from young plants were excised with sterile blade and collected in a beaker. The excised explants were thoroughly washed with running tap water for 10 - 15 minutes for removal of dust. Then they were washed with detergent (Teepol 5% v/v) solution for 3 minutes, fungicide (Bavistine 2%w/v) for 2 minutes then soaked in 70% (v/v) ethanol for 30 seconds and finally disinfected with 0.1% (w/v) HgCl2 for 2 minutes and rinsed with sterile distilled water five times.

2.3.3. Sterilization of Glasswares

The glasswares include culture tubes, conical flask, beakers, petridishes, pipettes, standard flask, and measuring cylinders etc. The glasswares were first soaked in sulphuric acid for four hours and then washed well under a jet of tap water. Then they were soaked
in detergent solution for 30 minutes and washed thoroughly using running tap water to remove the trace of detergent. Finally, they were rinsed with sterile distilled water, dried in a hot-air oven and autoclaved at 121°C for 20 minutes.

2.3.4. Preparation of Stock Solution

2.3.4.1. Preparation of Nutrients (Salts)

The stock volume of all ingredients varies depending upon the requirements and concentrations. Appropriate quantities of salts were weighed and dissolved in double glass distilled water. Macronutrients (Required Amount x 10) were dissolved separately and made up to the 1000 ml in brown bottle by mixing both solution and micronutrients (RA x 100). They were prepared as stock (100 ml final volume) solution. Iron source (RA x 100) stock solution was prepared as follows: FeSO$_4$.7H$_2$O and Na$_2$EDTA were dissolved and heated separately and made up to the (100 ml) final volume by mixing both solutions. Stock solutions were kept in sterilized brown bottles and stored in a refrigerator at 4°C and were used within 1 - 2 months from the date of preparation. To prepare one litre medium 100 ml macronutrients, 1.0 ml of micronutrients and iron stock solution were added before autoclaving.

2.3.4.2. Preparation of Vitamins and Amino acids

Pyridoxine HCl (50mg), nicotinic acid (50mg) and thiamine HCl (10mg) were dissolved separately and made to 100 ml of final volume by mixing both solutions. Myo inositol (100mg) was directly used during the one litre preparation of medium. Glycine (0.2g) was dissolved in distilled water and to made upto100 ml of stock solution. To prepare one litre medium, 1.0 ml of vitamins and amino acid stock solution were added before autoclaving.

2.3.4.3. Preparation of Hormones

Preparation of growth regulator stock solutions was as follows. Cytokinins víz., Kinetin (50mg) was dissolved in few drops of 0.1N HCl and diluted with distilled water to make up 1.0mg/1.0ml and stored in the refrigerator. The same concentration of adenine sulphate (AdS), thidiazuron (TDZ) and 6-benzylaminopurine (BAP) at 1.0mg/1.0ml was prepared like Kinetin. Auxins such as Indole-3-acetic acid (IAA) (50mg), Indole-3-butyric acid (IBA) (50mg) and α-Naphthaline acedic acid (NAA) (50mg) were dissolved
separately in few drops of 0.1N NaOH and diluted with distilled water to make up 1.0mg/1.0ml each and stored in the refrigerator. 2,4-Dichloro phenoxy acetic acid (2,4-D) (50mg) and Gibrellic acid (GA₃) (50mg) was dissolved separately a few drops of 70% ethanol and diluted with distilled water to make up 1.0mg/1.0ml each and stored in the refrigerator. Each growth regulators concentration has been kept as 1.0ml=1.0mg. These hormones were stable to be added before autoclaving the medium. They were prepared using general formula as shown below:

\[ \text{ML of medium x required hormone concentration} = \frac{\text{---------------------------------\vphantom{1000}}}{1000} \]

2.3.4.4. Selection of Medium and Carbohydrates

Different culture media namely MS (Murashige and Skoog, 1962), half strength MS, MS salts + B5 (Gamborg et al., 1968) vitamins and B5 (Gamborg et al., 1968) medium were tested for their suitability to get the maximum regeneration response. Carbohydrates like glucose, fructose, sucrose and maltose (10g/l - 50g/l) were supplemented to study shoot multiplication.

2.3.4.5. Preparation of Basal Medium (Solid and Liquid)

An aliquot of the frozen stock solution was thawed at room temperature just before use. To make up one litre of medium, about 500 ml of distilled water was added to a clean Erlenmeyer one litre standard flask. All the constituents of MS (1962) and B5 (Gamborg et al., 1968)) basal media as shown in Table - 2.1 and Table - 2.2, respectively. Required amounts of macronutrients, micronutrients, iron sources, vitamins and amino acids were pipetted out from stock solutions and sucrose (2 - 3%w/v) was added slowly to prevent clumping of the source in the bottom of the flask. Then, the hormones were added. It was then brought to the one litre volume and adjusted to pH 5.8 with 0.1N NaOH or 0.1N HCl. Agar (0.8% w/v) was dissolved in the medium by heating. The medium was distributed in 15 ml aliquots into 25 x 150 mm culture tubes or 50 ml aliquots into 250 ml of conical flask and plugged with non absorbent cotton wrapped and autoclaved at 121°C (1.06kg/sq.cm stream pressure) for 15 minutes. The racks of tubes were covered with clean plastic sheet to prevent deposition of dust and contamination. Preparation of liquid medium was similar to that of solid medium without agar.
2.3.5. Inoculation of Explants

The inoculation was done in a laminar air flow hood. Laminar air flow chamber was sterilized with 70% ethanol and by UV-irradiation for 15 minutes. The (in vitro viz., node, axillary bud and apical bud and in vivo viz., node, axillary bud and apical bud) explants were inoculated by vertical orientation on the medium containing different concentrations with combination of growth regulators. By means of a long stainless steel forceps, one explant per tube was placed. It was routine process to flame the mouth of the test tube after uncapping and before recapping the tubes to reduce contamination. To facilitate planting, two forceps were used alternatively to allow adequate time to cool, furthermore, to prevent burning the fingers and explants. Each treatment consisted of 7 explants and the experiments were repeated five times.

2.3.6. Culture Condition

All the cultures were maintained at 25 ± 2°C under 16/8 hrs light/dark condition of 80µEms-2s-1 irradiance provided by fluorescent lamps (TL 40W/54 cool-day light). In vitro response of inoculated explants was assessed every week in culture by counting the proliferated shoots which attained 2.0 cm in length and above. The subsequent subculture was made only on the medium containing maximum shoot proliferation rate.

2.3.7. Shoot Proliferation and Multiplication

Different (in vitro and in vivo) explants were cultured on MS basal medium containing 3% (w/v) sucrose, 0.8% (w/v) agar and various concentrations BAP and KN alone or in combination of BAP + GA₃/NAA and KN + GA₃/NAA were used for shoot proliferation. After two weeks the clumps of shoots were subcultured on MS medium containing 3% (w/v) sucrose and 0.8% agar (w/v) with suitable growth regulators for multiplication and maturation of the shoots. In addition to activated charcoal (0.5 - 3.0%) coconut water (10 - 15%) was added to promote the maximum number of shoots without microbial contamination.

2.3.8. Shoot Elongation

Proliferated multiple shoots were divided into small clusters of 2 - 3 shoots. They were subcultured on shoot elongation medium containing GA₃/KN (0.2 – 1.2mg/l) alone or in combination with cytokinins BAP/KN (0.1 - 2.5mg/l) or auxin NAA (0.1 - 2.5mg/l). The cultures were incubated at 25 ± 2°C under 16/8hrs light/dark photoperiod. After two weeks, shoots longer than 3.0 cm were counted and transferred to rooting medium.
2.3.9. Root Induction and Transplantation

The longer shoots (3.0 cm length) were excised and transferred to MS basal medium containing 3% (w/v) sucrose, 0.8% (w/v) agar and different concentration of IBA, and IAA (0.1 - 2.5mg/l) alone or in combination of cytokinins such as IBA + KN, IBA + BAP and IAA + KN (1.5 + 0.1 - 2.5mg/l) for root induction. Rooting was observed from 15 to 20 days. Plantlets with well developed roots were removed from the culture tubes and after washing their roots in running tap water, they were grown in the mixture of river sand, red garden soil and saw dust in the ratio of 1:1:1 in the paper cups for a month and subsequently transferred to pots. Potted plants were covered with transparent polythene membrane to high humidity and watered every three days with half strength MS salts solution free of sucrose for two weeks.

2.3.10. Acclimatization and Hardening of Regenerants

Plants raised through tissue culture, need gradual acclimatization for their survival in the outside condition, instead of transferring directly to the pots. Plantlets were left for a week in the paper cups at the controlled temperature (25 ± 2˚C) with 60% relative humidity. The survival percentage was observed in all the explants. After the initiation of new roots, they were kept in the green house and grown till maturity. Samples were photographed at different stages of growth period.

2.3.11. Statistical Analysis

2.3.11.1. Observation of Culture and Presentation of Results

The cultures were examined periodically and the morphological changes were recorded on the basis of visual observation. Whenever possible the effects of different treatments were quantified on the basis of percentage of cultures showing the response per culture. The experimental design was in Completely Randomized Design (CRD) and factorial with auxin and cytokinin as independent variables. Each treatment consisted of at least 7 explants and all the experiments were repeated five times. The data pertaining to frequency of shoot proliferation and multiplication, shoot elongation and root induction/culture were subjected to standard deviation. Mean separation was conducted by using Duncan (1955) new Multiple Range Test (DMRT) and means were compared with the P < 0.05 at level of significance.
2.3.11.2. Calculation

The experimental results were calculated as follows:

\[
\text{Frequency of shoot proliferation} : \frac{\text{No. of shoots proliferated}}{\text{No. of explants cultured}} \times 100
\]

\[
\text{Frequency of root induction} : \frac{\text{No. of shoots rooted}}{\text{No. of shoots cultured}} \times 100
\]

2.4. RESULTS

2.4.1. Initiation and Maintenance of Aseptic Culture

Explants isolated from field grown plants of *Plectranthus barbatus* encountered with microbial contaminations showed poor regenerative response. Stringent sterilization of culture medium had no effect and could not alleviative these problems. Hence *in vitro* raised plants were used as explant sources for the present investigation. Among the various sterilizing agents used for the sterilization of explants, exposure to 3 minutes treatment with detergent solution (Teepol 5% v/v), 70% (v/v) ethanol for 30 seconds and 0.1% (w/v) HgCl\(_2\) for 2 minutes was found to be effective for maximum response (90%) with minimum contamination (10%). Sterilized explants were inoculated on the MS basal medium supplemented with cytokinins and auxins alone or with combination.

The meristem containing apical bud, axillary bud and node (both *in vitro* and *in vivo*) explants were cultured on MS basal medium without growth regulators survived only for a few days and they shriveled off. Organogenic potential of apical bud, axillary bud and node explants were tested in MS medium containing cytokinins (BAP and KN) gibberelic acid (GA\(_3\)) and auxins (IBA, IAA and NAA) individually or in combination with each at different concentrations. The MS nutrient medium incorporated with cytokinins and auxins individually or in combinations promoted either shoot bud formations/callus or rhizogenesis with their optimal results and they were tabulated. Explants maintained polarity and had a tendency of producing callus, when inoculated horizontally on the surface of the medium. On the other hand, the explants when placed vertically under suitable hormonal combination responded favorably to shoot organogenesis.
2.4.2. Shoot bud Proliferation

2.4.2.1. Media Response

Different media such as MS, half strength MS, MS salts + B5 vitamins and B5 media containing different concentrations of KN alone or in combination with GA₃ were tested for their influence on multiplication. Of these four media used, MS medium with KN (1.0mg/l) and KN + GA₃ (1.0/1.5 + 0.6mg/l) showed good response in all the explants and higher frequency of shoot proliferation (Table - 2.3). Among the four media used, MS medium showed optimum shoot bud proliferation from *in vitro* apical bud, axillary bud, *in vivo* apical bud, axillary bud, *in vitro* node and *in vivo* node explants when compared to other media. B5 media had lower effect of shoot bud proliferation, MS salts + B5 vitamins and half strength MS media showed moderate effect of shoot bud proliferation (Fig. - 2.1).

2.4.2.2. Effect of Carbohydrates

Among the four different sugars (glucose, fructose, sucrose and maltose) that were tested, sucrose showed maximum response to multiple shoot induction. It was followed by glucose and fructose. Maltose was inefficient in inducing multiple shoots from both *in vitro* and *in vivo* derived explants (Table - 2.4). To find out the optimum level of sucrose requirement, different levels of sucrose (10 – 50g/l) were tested in MS medium. Three percent sucrose (30g/l) level was the most suitable for multiple shoot induction. An increase of 3% or a decrease of 3% concentration of sucrose resulted in poor shoot multiplications (Fig. - 2.2).

2.4.2.3. Efficiency of Growth Regulators

Various growth regulators were tested for the shoot bud proliferation from various explants *viz.*, *in vitro* apical bud, axillary bud and node and *in vivo* apical bud, axillary bud and node on MS medium supplemented with different concentrations of either (BAP and KN) alone or in combination with GA₃/NAA. MS medium containing BAP alone induced shoot proliferation with low frequency, whereas KN alone or combination with GA₃ increased percentage of response, but combination of KN or BAP with NAA showed minimum frequency of response than BAP for 25 days with two subcultures on the same medium. The highest percentage of culture response and optimal number of shoots from different explants of micropropagation is given in Fig. – 2.3.
2.4.3. Explant Culture

It was observed that after 7 days of inoculation, the explants enlarged 2 - 3 times of the original size. All the six explants (in vitro apical bud, axillary bud and node and in vivo apical bud, axillary bud and node) cultured on free hormonal medium, did not show any sign of shoot bud development after fifteen days of culture. The addition of cytokinins and auxins seemed to promote bud proliferation and enhanced shoot bud development in all explants. Among the various explants tested, in vitro apical bud and axillary bud showed maximum frequency of shoot proliferation response with the highest number of shoot formation (Table - 2.3). Following main steps in regeneration of plants through micropropagation is given in Fig. – 2.4.

2.4.3.1. In vitro Apical bud

The morphogenesis response of in vitro apical bud explants as was observed on 0.1 to 2.5mg/l for KN, BAP, KN + GA₃, BAP + GA₃ and KN + NAA are summarized in Table - 2.5. Among the various cytokinins tested in MS medium, KN was found to be more efficient than others with respect to initiation and subsequent proliferation of shoots. All the concentrations of KN/BAP (0.1, 0.5, 1.0, 1.5, 2.0 and 2.5mg/l) alone facilitated shoot buds differentiation. Of the various levels of KN and BAP that were tested, KN 1.0mg/l proved to be the most effective on MS medium and an average of 11.63 ± 2.17 shoots were developed per explant in 91.4% of cultures, followed by BAP 1.5mg/l and showed an average of 10.11 ± 2.56 shoots developed/explant with 80% response (Plate - 2.1). An increase or decrease of concentrations of each cytokinin, showed a reduction in the number of shoots per culture. A callus occasionally was formed at the base of explant, retarding bud formation and subsequent growth of shoots. Therefore, precautions were taken to remove such callus growth while subculturing.

The efficiency of the optimal concentration of KN and BAP with GA₃ or NAA was also evaluated for multiple shoot induction. KN with GA₃ was found to be the most effective combination for shoot regeneration and multiplication. In vitro derived apical bud cultured on MS medium supplemented with KN 1.0mg/l + GA₃ 0.6mg/l exhibited 85.7% shoot regeneration with an average of 11.00 ± 2.89 shoots per explant, followed by BAP 1.5mg/l + GA₃ 0.6mg/l which showed 80% shoot regeneration with an average of 7.21 ± 1.55 shoots per explant. Minimum shoots were observed when the explant was
cultured on MS medium containing KN + NAA (1.0 + 0.6mg/l). Upon increasing concentrations of GA\textsubscript{3} or NAA up to 1.2mg/l, a gradual decrease in regeneration frequency and number of shoots per explant was recorded. The elevated concentrations of NAA 1.2mg/l resulted in little callusing at the cut end thus reducing the percentage of shoot regeneration and number of shoots per explant (Table - 2.5).

2.4.3.2. \textit{In vitro} Axillary bud

Multiple shoots were induced from \textit{in vitro} derived axillary bud cultured on MS medium supplemented with different concentrations of cytokinins (KN and BAP) or in combinations with GA\textsubscript{3}/NAA. A maximum of 10.89 ± 2.60 shoots per explant with 88.6% frequency developed on MS medium fortified with KN (1.0mg/l) within 20 days of inoculation (Table - 2.6). The same medium with BAP (1.5mg/l) also produced the highest multiplication of 9.58 ± 1.89 shoots per explant with 82.9% frequency. The rate of shoot multiplication was significantly enhanced by the combinations of KN + GA\textsubscript{3} (1.0 + 0.6mg/l). The best interaction, giving the highest rate of shoot multiplication 10.79 ± 1.84 was found to be with 85.7% frequency. The optimum (6.58 ± 2.09) shoot proliferation was obtained on MS medium containing BAP + GA\textsubscript{3} (1.5 + 0.6mg/l), followed by KN + NAA (1.0 + 0.6mg/l) with 6.00 ± 1.49 shoots per explant. It therefore, appeared that KN + NAA have synergistic effect on shoot multiplication. Increased NAA concentration resulted in decreased shoot multiplication and basal callus induction (Plate - 2.2).

2.4.3.3. \textit{In vivo} Apical bud

The effect of different concentrations of KN or BAP (0.1 – 2.5mg/l) and combinations with GA\textsubscript{3} or NAA (0.2 - 1.2mg/l) on shoot organogenesis are presented in Table - 2.7. Besides attaining a maximum number of responding explants with the highest frequency (88.6%) the maximum number of shoots (10.79 ± 2.02 shoots) per explant was attained on KN (1.5mg/l) + GA\textsubscript{3} (0.6mg/l). This was followed by KN (1.5mg/l) alone and maximum of 88.6% of responding with an average of 10.68 ± 2.31 shoots per explant. BAP (1.5mg/l) alone or combination with GA\textsubscript{3} (0.6mg/l) resulted in optimum shoots production frequency (82.9% and 71.4%) with shoot multiplication (9.89 ± 1.88 and 7.05 ± 1.78), respectively. Minimum shoot regeneration frequency (65.7%) and mean shoot number 5.95 ± 1.51 were obtained with induction media supplemented with 1.5mg/l KN and 0.6mg/l GA\textsubscript{3} (Plate - 2.3).
2.4.3.4. **In vivo Axillary bud**

*In vivo* axillary bud was cultured on MS medium fortified with KN or BAP (1.0 – 2.5mg/l) alone or in combinations with GA\(_3\)/NAA (0.2 – 1.2mg/l) for multiple shoot induction. After 15 days, the axillary bud growth initiation was observed and bud developed into shootlets within 20 days. Of two cytokinins used, KN (1.5mg/l) and BAP (1.5mg/l) gave 85.7% and 80% shootlet formation and average number of 10.26 ± 2.35 and 9.05 ± 1.75 shoots per explant. The best response in the terms of shoot regeneration and biomass increase was observed on MS medium enhanced with KN (1.5mg/l) + GA\(_3\) (0.6mg/l). An average number of maximum shoots (10.47 ± 1.87 shoots) per explant was scored with 88.6% of frequency. Among the BAP + GA\(_3\) and KN + NAA combination, the minimum frequency (68.6% and 62.9%) shoot bud formation and lower number of shoots (6.84 ± 1.64 and 5.63 ± 1.21) per explant were obtained on MS medium containing BAP + GA\(_3\) (1.5 + 0.6mg/l) and KN + NAA (1.5 + 0.6mg/l) (Table - 2.8 and Plate - 2.4).

2.4.3.5. **In vitro node**

Multiple shoot formation from *in vitro* derived node explants was observed from 0.1 – 2.5mg/l concentrations of KN and BAP individually or in combination with GA\(_3\)/NAA (0.2 – 1.2mg/l). Maximum percentage of response in different hormones were 88.6% for KN, 82.9% for BAP, 85.7% for KN + GA\(_3\), 71.4% for BAP + GA\(_3\) and 65.7% for KN + NAA. Among the growth regulators KN 1.0mg/l + GA\(_3\) 0.6mg/l showed maximum (9.11 ± 2.02) number of shoots and minimum (3.11 ± 0.94) with KN at 1.0mg/l + GA\(_3\) 1.2mg/l (Table - 2.9). Addition of BAP + GA\(_3\) had enormous effect on inducing multiple shoots. BAP (1.5mg/l) and GA\(_3\) (0.6mg/l) induced minimum (5.95 ± 1.58) shoots followed by KN (1.0mg/l) and NAA (0.6mg/l) induced basal callus with minimal (5.58 ± 1.22) shoots (Plate - 2.5).

2.4.3.6. **In vivo node**

A number of treatments were tried to regenerate shoots from node explants that were excised from *in vivo* field grown plant. A number of explants responded to regenerate multiple shoots in different concentrations of KN/BAP with GA\(_3\)/NAA. The highest percentage of node explants regenerated shoots was 85.7% in MS medium containing 1.0mg/l KN with 0.6mg/l GA\(_3\) after 20 days of subculture, the highest mean number of shoots 8.47 ± 1.47 per explant were recorded. Optimum shoot regeneration
occurred on MS medium having 1.0mg/l for KN (7.79 ± 1.32) with 82.9% responsive culture followed by 1.5mg/l for BAP (7.11 ± 1.10) 80% responsive culture. Lowest percentage of regenerating shoots from nodal segment was 65.7% and 62.9% in MS medium supplemented with 1.5mg/l BAP + 0.6mg/l GA₃ and 1.0mg/l KN + 0.6mg/l NAA, respectively. The lowest mean number 5.63 and 5.26 shoots per explant were recorded in these same media compositions (Table - 2.10 and Plate - 2.6).

2.4.4. Shoot Elongation

For shoot elongation, 30 days old multiple shoot mass were transferred to half strength MS medium containing of GA₃/KN (0.2-1.2mg/l) alone or various combinations with different concentrations of KN, BAP and NAA (0.2 - 1.2mg/l). Among the various combinations used, GA₃ 0.6mg/l + KN 0.5mg/l combination supported maximum shoot length (8.36 cm length/shoot) and mean number of average node 8.11 with 16.2 leaves/explant within 20 days of culture (Plate - 2.1.e) (Table - 2.11). When increasing the concentration of GA₃ the shoot length also increased up to optimum level (0.6mg/l), afterwards it decreased. The optimum concentration of GA₃ (0.6mg/l) + BAP (1.0mg/l) or NAA 0.5mg/l also proved best for shoot elongation, and further increase of BAP (1.0mg/l) and NAA (0.5mg/l) enhanced the multiple shoots or basal callusing. Optimum concentration of KN at 0.6mg/l alone also enhanced shoot elongation.

2.4.5. Rooting of in vitro shoots

Well developed shoots (above 3 cm) were excised and grown in half MS medium supplemented with either IBA or IAA (0.1 - 2.5mg/l) in combination with KN or BAP (0.1 - 2.5mg/l). The 15 days of culture in rooting medium resulted in maximum percentage of rooting and 4 to 6 days of dark treatment stimulated maximum rooting in IBA alone or IBA + KN supplemented medium. The percentage of rooting increased with the increasing concentrations of IBA/IAA upto 1.5mg/l and it decreased with further increase of IBA/IAA. The hormone concentration with respect to percentage of rooting per shoot and nature of roots were controlled by auxins and their concentrations.

a. Effect of IBA

Shoots were subcultured into half strength MS medium with different concentrations of IBA (0.1 - 2.5mg/l) individually for rooting. Among the various concentrations used, IBA at 1.5mg/l was found to be with higher percentage of rooting in
both *in vitro* and *in vivo* derived shoots (Table - 2.12). Of the different explants tested, *in vitro* node derived shoots produced higher frequency of rooting (85.7%) response with 20.5 roots/shoot and an average of maximum root length (9.16 cm) were observed within 15 - 20 days of culture (Plate - 2.1.e and 2.7.b).

### b. Effect of IAA

*In vitro* raised shoots were transferred to half strength MS medium fortified with various concentrations of IAA (0.1 - 2.5mg/l) for rooting. Among the various concentrations of IAA used, 1.5mg/l of IAA was found to be the optimal for maximum rooting response. The maximum (71.4%) frequency of rooting was noticed, when the shoots were cultured in half MS medium fortified with optimum level of IAA (1.5mg/l) and produced 14.1 roots/shoot with 6.16 cm root length.

### c. Effect of Auxins with Cytokinins

The optimum concentration of IBA/IAA 1.5mg/l for rooting selected in the previous experiment was tested in combination with various concentrations of KN/BAP (0.1 - 2.5mg/l). The combination of IBA 1.5mg/l + KN 0.5mg/l showed maximum percentage (80%) response with 17.1 roots and average of 8.63 cm root length, followed by IBA (1.5mg/l) + BAP (0.5mg/l) showed 14.4 roots with 7.16 cm root length. Less number of (8.21) roots with 5.11 cm root length was obtained when culturing *in vitro* regenerated shoots on half MS medium containing with IAA (1.5mg/l) + KN (0.5mg/l) as shown in the Table - 2.12.

### 2.4.6. Acclimatization and Hardening

Rooted plantlets of 2 weeks old cultures were transferred to soil under shade for *in vitro* hardening. The plantlets were taken out from the flasks, washed with sterile water removed agar/medium and transferred to plastic cups containing river sand, garden soil and saw dust in the ratio of 1:1:1. These plantlets were treated with half strength medium twice for 20 days in controlled conditions and covered with polythene bags (Plate - 2.1 to 2.6).

The plants were maintained under controlled temperature (25 ± 2°C) for a week subsequently they were transferred to the field and successfully established in the nursery bags, then transferred to the field (Plate - 2.7). The regenerated plants did not show any detectable variation in morphology or growth characteristics with the respective donor plants. The survival percentage was 90 - 95%.
2.5. DISCUSSION

In vitro micropropagation is an effective means for rapid multiplication of species with a high progeny uniformity. Therefore, there was the interest in using this technique for rapid and large-scale propagation of medicinal plants. Many in vitro studies have been conducted on members of Lamiaceae including Ocimum, using different explants like nodal and axillary bud (Begun et al., 2000), leaf (Phippen and Simon, 2000) and young inflorescence (Singh and Sehgal, 1999). Reports of in vitro regeneration from tissue of Lamiaceae members are available in Coleus forskohlii (Reddy et al., 2001), Cuniliagalioides (Fracaro and Echeverrigaray, 2001), Ocimum basilicum (Dode et al., 2003; Sudhakaran and Sivasankari, 2003), Salvia canariensis (Molina, 2004), Pogostemon cablin (Parida et al., 2005), Mentha piperita (Shasany et al., 2006), Ocimum gratissimum (Gopi et al., 2006), Teucrium stocksianum (Bouhouche and Ksiksi, 2007), Basilicum polystachyon (Amutha et al., 2008) and Salvia africana-lutea (Makunga and Staden, 2008).

The present study was undertaken to develop an efficient protocol for micropropagation from both of in vitro and in vivo explants of Plectranthus barbatus. The observation showed that explants in their response with respect to hormone and its combinations for shoot multiplication, shoot elongation, rooting and hardening process.

2.5.1. Initiation and maintenance of aseptic culture

The significant part of this study was the propagation of contamination-free plants. Explants like apical bud, axillary bud and node from the natural population were sterilized with 70% ethanol and 0.1% HgCl₂ for two minutes and they were effective in controlling the fungal and bacterial contamination without harming the explants. These results corroborate the earlier observation made in Ocimum basilicum (Dode et al., 2003), Bambusa glaucescens (Shirin and Rana, 2007), Brasenia schreberi (Oh et al., 2008), Basilicum polystachyon (Amutha et al., 2008), Daphne giralldii (Noshad et al., 2009) and Quercus rubra (Vengadesan and Pijut, 2009). The use of medium sized explants had higher survival and growth rates than smaller ones (Jayaram and Prasad, 2007). In vitro derived apical bud, axillary bud and node explants showed results of shoot multiplication rates that were higher than in vivo field grown explants. Similarly, many other results have also recommended the use of apical bud explant as in Drosera peltata (Kim and Jang,

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2004) and *Sorghum bicolor* (Baskaran *et al*., 2006), axillary bud explant of *Centella asiatica* (Tiwari *et al*., 2000) and *Paeonia suffruticosa* (Beruto *et al*., 2004) and node explant of *Sophora flavescens* (Zhao *et al*., 2003), *Sesbania drummondii* (Cheepala *et al*., 2004) and *Wattakaka volubilis* (Chakradhar and Pullaiah, 2006).

2.5.2. Shoot bud Proliferation

2.5.2.1. Effect of Media

According to Shirin and Rana (2007) and Chaudhuri *et al*., (2008), the best medium for *in vitro* multiplication of *Plectranthus barbatus* is MS medium. In most of the herbaceous plant species *in vitro* multiplication is very well in MS medium has been used. MS salts along with B5 vitamins for the *in vitro* regeneration studies have been reported (Molina, 2004 and Sharma *et al*., 2006). The concentration of inorganic nutrients is higher in MS medium than other media while vitamin level is more in B5 medium. Sharma *et al*., (2006) and Anand *et al*., (2001) reported that MS + B5 medium had greater effect and improved the *in vitro* culture studies in *Vitex negundo* and *Vigna unguiculata*, respectively. The present study has evolved an efficient and reproducible method to obtain a high frequency of shoot from culturing full strength MS medium. Similar observations were reported by many authors (Tiwari *et al*., 2002; Mahesh and Jeyachandran, 2005; Sujatha and Ranjithakumari, 2007; Chaudhuri *et al*., 2008 and Amoo *et al*., 2009). In this study it is seen that MS medium showed optimum frequency of response which produced more number of shoots with shoot length. The other media showed minimum frequency of response which produced very less numbers of shoots with shoot length.

2.5.2.2. Effect of Carbohydrates

Among the different sugars used, sucrose induced the initiation of maximum number of shoots. Glucose and fructose gave satisfactory results while maltose was inefficient in inducing multiple shoots. According to Lakshmiprabha *et al*., (2008) and Unda *et al*., (2007) autoclaved sucrose is better than filter sterilized sucrose for shoot proliferation as hydrolysis of sucrose during autoclaving led to the formation of readily utilizable glucose and fructose for growing tissues, on the other hand autoclaved fructose is not advisable as it could be detrimental to growing tissue. In conformity with the previous reports, autoclaved sucrose was found to be suitable for *Plectranthus barbatus* in the present study also.
2.5.2.3. Effect of plant growth Regulators

In this study shoot initiation was observed, when the explants were cultured in medium with plant growth regulators. Therefore, optimum concentration of cytokinins (KN or BAP) responded effectively. Optimum concentration of KN (1.0mg/l) or BAP (1.5mg/l) alone was effective for shoot multiplication in *Plectranthus barbatus*. Similar observation was reported in *Ocimum gratissimum* (Gopi et al., 2006) and *Basilicum polystachyon* (Amutha et al., 2008) and KN (1.0mg/l) combination with GA$_3$/NAA (0.6mg/l) promoted shoot multiplication and shoot elongation. Similar response was also observed in other species as in *Momordica charantia* (Huda and Sikdar, 2006) and *Symonathus bancroftii* (Panaia et al., 2000).

2.5.3. Explant Culture

Among the different explants such as *in vitro* derived apical bud, axillary bud and node and *in vivo* field grown explants like apical bud, axillary bud and node, *in vivo* field explants and *in vitro* derived explants of *Plectranthus barbatus* which were cultured on MS medium fortified with various concentrations of cytokinins (KN or BAP) either singly or in combination with GA$_3$/NAA. KN alone or combination with GA$_3$ proved best for inducing healthy shoots in *in vitro* apical bud, axillary bud and node followed by *in vivo* apical bud, axillary bud and node less effective shoot proliferation than *in vitro* explants. The efficiency of NAA in inducing optimum shoot number along with cytokinin in both *in vitro* and *in vivo* nodal explants is well supported by Anitha and Pullaiah (2002) and Chakradhar and Pullaiah (2006).

2.5.4. Shoot Proliferation and Multiplication

Fundamentally, vegetative multiplication is the result of mitosis or division of cells without altering the genetic make up of the plant. This can be extended to the multiplication of plant organs. The present study has been successful in evolving a protocol for *in vitro* proliferation of multiple shoots and plantlets development for shoot bud proliferation and multiplication.

The major properties of cytokinins useful in culture are the stimulation of cell division and release of lateral bud dormancy. The most commonly used cytokinins are BAP and KN. Cell division is regulated by the joint action of Auxins, cytokinins and
Gibberellic acid, each of which influence different places of cell cycle. The variations in the regeneration potential among explants are attributable to the differences in their physiological and genetic make up of cells.

2.5.4.1. In vitro Apical bud

Explants were capable of directly developing multiple shoots on MS basal medium containing different concentrations of cytokinins (Amutha et al., 2008). In the present study multiple shoot formation from apical bud collected from in vitro explant, was obtained on MS medium supplemented with KN (1.0mg/l). Similar results of KN (1.0mg/l) showed maximum frequency and multiple shoots in Ocimum gratissimum (Gopi et al., 2006). Various concentrations of BA were helpful in inducing the growth of apical bud. However, the mass and rapid growth was observed on MS medium with optimum concentration of BAP (1.5mg/l). Ahmed et al. (2000) reported that treatment MS + BA and MS + KN showed good responses for shoot initiation and elongation in tomato. Similar results were reported in a genus of related family (Lamiaceae) Salvia with a minimum concentration of BAP (1.0mg/l) for shoot regeneration (Makunga and Staden, 2008). A further, increase of concentration of BAP had no effect on the number of multiple shoots as in Curculigo orchioides as reported (Wala and Jasrai, 2003). The combination of KN + GA3 yielded maximum number of shoots proliferation as compared to individual affected by BAP. Thus, the KN (1.0mg/l) and GA3 (0.6mg/l) combination was found to be efficient for maximum frequency of shoot proliferation and multiplication. These results are in agreement with those previously reported by Huda and Sikdar (2006) and that the combination of GA3 + KN was best for developing shoots in Momordica charantia.

2.5.4.2. In vitro Axillary bud

Proliferation of multiple shoots was obtained with higher frequency from in vitro axillary bud explants. These explants were capable of directly developing multiple shoots on MS medium containing different concentrations of cytokinins. Multiple shoot initiation was observed from the explant in KN and BAP fortified media within 15 to 20 days after inoculation. The highest number of shoots were observed in the medium containing KN (1.0mg/l) followed by BAP (1.5mg/l) and similar observation was made by Jawahar et al. (2004) in Solanum trilobatum, Verma and Kant (1996) in Emblica officinalie and Deka
et al. (1999) in *Withania somnifera*. Kulkarni and Rao (1999) reported that KN did not support the proliferation of multiple shoots in *Acorus calamus*. High level of cytokinins stimulate callus formation and inhibit shoot proliferation as observed in elm species (McCown and McCown, 1987; Corchete et al., 1993 and Thakur and Karnosky, 2007). Among the cytokinins, GA$_3$ or NAA enhanced maximum number of shoot multiplication as observed in *Wattakaka volubilis* (Chakradhar and Pullaiah, 2006), *Hemidesmus indicus* (Sreekumar et al., 2000), *Cayratia petata* (Anupama et al., 2005), *Centella asiatica* (Tiwari et al., 2000) and *Momordica charantia* (Huda and Sikdar, 2006). This is analogous to the present study, where optimum number of shoot proliferation was observed in MS medium with KN (1.0mg/l) + GA$_3$ (0.6mg/l) or BAP (1.5mg/l) + GA$_3$ (0.6mg/l).

2.5.4.3. *In vivo* Apical bud

Multiple shoot induction was found to be higher in *in vivo* apical bud explant cultured on MS medium augmented with different concentrations of KN and BAP alone. The highest number of shoot multiplication was obtained on MS medium with KN (1.5mg/l) followed by BAP (1.5mg/l) and produced less number of shoots. Similar effect of KN and BAP were reported by Gopi et al. (2006), Faisal et al. (2007) and Amutha et al. (2008). Soniya and Das (2002a) reported that shoot tips cultured on MS medium supplemented with KN and BAP developed multiple shoots after 20 days. Kabir et al. (2006) reports that KN and BA either alone or combination promoted shoot formation and its multiplication as in *Thuja occidentalis*. These data were similar to those of Kim and Jang (2004) and Sebastian et al. (2006a) who found that the best multiplication on KN than BAP. Cytokinin alone or in addition of GA$_3$ has been extensively used in primary establishment of tomato meristens (Ahmed et al., 2000). The high percentage of primary establishment of meristem of brinjal was observed on MS + BAP by Nasir (2004). In the present study a callus free micropropagation system producing large number of plants from *in vivo* apical bud collected from field grown plants has been evolved.

2.5.4.4. *In vivo* Axillary bud

Mass propagation of plant species through *in vitro* culture of plant tissue culture are successful examples of commercial applications of plant tissue culture technology. Recently, there has been much progress in this technology for conservation for genetic
resources and clonal improvement (Wawrosch and Kopp, 1999; Cuenca and Marco, 2000; Hassan and Roy, 2005; Loc et al., 2005; Gavhane and Mukundan, 2006; Chaudhuri et al., 2008 and Amoo et al., 2009). Rapid shoot regeneration has been achieved with axillary bud of field grown herbaceous medicinal plant species (Hall and Champer, 2002; Beruto et al., 2004 and Jayaram and Prasad, 2007). The frequency of responding explants increased markedly with an increase in the concentration of KN/BAP up to 1.5mg/l and in combination with KN/GA3/NAA substantially increased in the frequency of responding axillary bud segments. Similar results were observed from axillary bud in Gloriosa superba (Hassan and Roy, 2005; Tiwari et al., 2000 and Huda and Sikdar, 2006). In this present case, KN (1.5mg/l) alone or combination with GA3 (0.6mg/l) also proved to be more effective than other cytokinins.

The synergistic effect of cytokinins in combination with an auxin has been demonstrated in many medicinal plant species, such as Ceropegia candelabrum (Beena et al., 2003), Bambusa glaucescens (Shirin and Rana, 2007), Centaurea spachii (Cuenca and Marco, 2000), Acorus calamus (Gavhane and Mukundan, 2006) and Curcuma zedoaria (Loc et al., 2005).

2.5.4.5. In vitro Node

In vitro proliferated micronodes were further used as source of explants. The recurrent production of nodes using explants from micronodes has been reported in Syzygium cuminii (Jain and Babbar, 2000), Pistacia vera (Onay, 2000) Poulownia tomentosa (Rout et al., 2001) and Crataeva nurvula (Walia et al., 2007). Walia et al. (2007) reported that compared to the nodal explants derived from field plant, micronodes from in vitro derived microshoots exhibited higher regeneration response. This study was in accordance with the present reports where MS medium containing KN (1.0mg/l) showed maximum (88.6%) percentage of response and maximum number of shoots. Jawahar et al. (2004) reported that higher number of shoots (36 shoots/explant) was observed in the medium containing BA (2.0mg/l) followed by KN (2.0mg/l) with 29 shoots. Fracaro and Echeverrigaray (2001) and Nagesh (2008) also reported that BAP produced maximum number of shoot than KN. This is in contrast to the present investigation, where maximum number of shoot proliferation (9.95 shoots/explant) was
observed in MS medium with KN (1.0mg/l) followed by BAP (1.5mg/l) with 8.47 shoots. These results corroborate with the previous finding in similar families with members of *Ocimum basilicum* (Sudhakaran and Sivasankari, 2003). GA₃ alone or in combination with cytokinins has been extensively used for primary establishment of meristem culture, as reported by Morel and Martin (1952) and Huda and Sikdar (2006). Thus, these results are analogous to the present observation, where KN along with GA₃ induced multiple shoots from *in vitro* node explant.

2.5.4.6. *In vivo* Node

Fracaro and Echaverrigaray (2001) described that the highest number of adventitious buds were produced in the presence of cytokinins. Cytokinin overcomes the apical dominance, releases lateral buds from dormancy and promotes shoot formation (George, 1993). Similar results were also reported in *Musa sapientum* (Akbar and Roy, 2006) and *Eclipta alba* (Ray and Bhattacharya, 2008), whereas among three cytokinins like BAP, KN and TDZ, BAP was the most effective in proliferating adventitious shoots. Similar results were observed in present study, but KN (1.0mg/l) proved the most suitable for shoot proliferation than BAP which was less effective for shoot bud multiplication. Hussein *et al.* (2005) also reported the direct plant regeneration from *in vivo* shoot tip on MS medium with KN (5.0mg/l). The inclusion of KN and addition of GA₃ (Panaia *et al.*, 2000 and Huda and Sikdar, 2006) and addition of NAA (Govindaraju *et al.*, 2003; Akbar and Roy, 2006), BAP + NAA (Hassan and Roy, 2005; Alderate *et al.*, 2006) combination induced high frequency of shoot proliferation. Similar results were obtained in the present investigation, where KN (1.0mg/l) + GA₃ (0.6mg/l), KN (1.0mg/l) + NAA (0.6mg/l) was effective in inducing multiple shoot from *in vivo* node explants, showing the efficiency of NAA in including optimum shoot numbers along with cytokinins in both *in vitro* and *in vivo* nodal explants. There results corroborate the previous work (Anitha and Pullaiah, 2002 and Chakradhar and Pullaiah, 2006).

2.5.5. Shoot Elongation

Regenerated shoots were sub cultured on half strength MS medium with the supplementation of GA₃ and KN for shoot elongation. GA₃ (0.6mg/l) individually showed maximum (88.6%) response, but the microshoots were unhealthy thin and leaf was pale
yellowish in colour. However, addition of KN in combination with GA\textsubscript{3} showed quick elongation and internodes elongation as well as healthy shoots with green leaves. Maximum shoot elongation was obtained on half MS medium containing KN + GA\textsubscript{3} in *Symonanthus bancroftii* by Panaia *et al.* (2000). In the meantime the concentration of KN produced a similar pattern in terms of stem elongation as was reported by Hussein *et al.*, (2005). Anand and Jeyachandran (2005) reported that shoot elongation was simultaneously observed along with root induction in NAA (2.0mg/l) in MS medium. Well developed microshoots were separated individually and carefully transferred for shoot elongation medium fortified with BAP (1.0mg/l) as reported by Mahesh and Jeyachandran (2005). This result corroborated with the previous finding in *Withania somnifera* (Govindaraju *et al.*, 2003) and *Jatropha curcas* (Purkayastha *et al.*, 2010). Many authors have observed shoot elongation using KN in *Ocimum basilicum* (Sudhakaran and Sivasankari, 2003), KN + Zn in *Cinnamomum camphora* (Babu *et al.*, 2003), BAP + GA\textsubscript{3} in cotton (Divya *et al.*, 2008) KN + BAP in *Sorghum bicolor* (Baskaran and Jayabalans, 2005) and *Enicostemma hyssopifolium* (Seetharam *et al.*, 2002), BAP + NAA in *Huernia hystrix* (Amoo *et al.*, 2009), *Tectona grandis* (Tiwari *et al.*, 2002) and *Tylotopora indica* (Faisal *et al.*, 2007). BAP only in *Onobrychis viciifolia* (Sancak, 1999) and *Artemisia vulgaris* (Sujatha and Ranjithakumari, 2007) and IAA in *Sesbania drummondii* (Cheepala *et al.*, 2004). In the present study GA\textsubscript{3} + KN (0.6 + 0.5mg/l) was found to be more potent to shoot elongation (8.36 cm shoot length) followed by GA\textsubscript{3} (0.6 mg/l) alone with 7.58 cm shoot length.

2.5.6. Rooting of *in vitro* Shoots

Rooting of shoots is the most critical step in the production of complete plantlets and their subsequent survival. Growth regulators and nutrient contents of the medium play a vital role in the rooting process. Both IBA and IAA were used individually and in combination with KN/BAP for rooting. In the present study IBA was found to be more potent for auxins for the highest percentage of rooting when compared to IAA. For rooting of *in vitro* raised shoots, the half strength MS medium with IBA promoted maximum rooting (Jha *et al.*, 2004; Tiwari *et al.*, 2000; Sancak, 1999; Bouhouche and Ksiki, 2007 and Faisal *et al.*, 2007), IAA for rooting (Seetharam *et al.*, 2002), half strength NAA for rooting (Wala and Jasrai, 2003).
Thus, these results are analogous to the present observation whereas half strength MS medium containing IBA (1.5mg/l) produced highest (20.5) roots with root length 9.16 cm followed by IAA at 1.5mg/l produced 14.11 roots with 6.16 cm root length. Nagesh (2008) has recorded that in vitro shoots were rooted on half strength MS liquid medium containing 1.0mg/l IBA and produced a greater number of healthy and sturdy roots than those cultured with NAA. Among the auxins used, IBA was found to be the best for root induction as reported by Akbar and Roy (2006), Kabir et al. (2006), Anupama et al. (2005), Gavhane and Mukundam (2006), Cheepala et al. (2004) and Fracaro and Echeverrigaray (2001). NAA was found to be more effective for root formation, whereas in Ocimum basilicum (Sudhakaran and Sivasankari, 2003), an addition of IAA (0.5mg/l) with IBA (1.0mg/l) supplementation on half strength MS medium produced 90 percent, when culturing single shoot. Our results indicated that the addition of cytokinins with IBA induced optimum number of roots.

2.5.7. Acclimatization and Hardening

The period of transition during the process of hardening after transfer from the in vitro to ex vitro environment is considered to be the most important step in tissue culture (Faisal et al., 2007). Tissue cultured plants lose their water rapidly, when moved to the external condition. In vitro plant leaf was very typical and incapable of complete stomatal closing under conditions of low relative humidity. For acclimatization plantlets were removed from rooting medium after two weeks at incubation and transferred to paper cups containing autoclaved soil tightly covered with perforated polythene bags to maintain (25 ± 2°C) humidity and were kept under culture room conditions for one week. This present study is consistent with the reports of Banu and Handique (2003) in Phyllanthus fraternus and Gopi et al. (2006) in Ocimum gratissimum. During hardening, the plantlets were irrigated with half strength MS medium and tap water when required. Similar results were also reported by Anupama et al. (2005), Wala and Jasrai (2003), Datta et al. (2007) and Evenor and Reuveni (2004).

Polythene bags were removed and plantlets were allowed to remain in pots for another one week, before they were transferred to large pots and irrigated with tap water, then hardened and acclimatized plants were directly planted in the field. Plantlets were
fully acclimatized at the time of transferring them to pots and no further losses were recorded thereafter. Three months later all the *in vitro* derived plants were still alive and displayed normal development similar to non-cultured plants, as reported by Makunga and Staden (2008).

The present study described an efficient and successful micropropagation system for production of plants in large-scale from *in vitro* and *in vivo* explants of *Plectranthus barbatus*. The various factors like different culture media, culture conditions and growth regulators were optimized to obtain maximum response. The protocol standardized in the present investigation is reproducible and can be used in future improvement program.