CHAPTER - IV

INDIRECT ORGANOGENESIS

4.1. INTRODUCTION

Higher plant body is multicellular and is made up of highly organized and differentiated structures like stem, leaf, root etc. Different tissue systems present in different organs function in a highly coordinated manner. Now, if the organized tissues are diverted into an unorganized proliferative mass of cells by means like mechanical, chemical or effective agents, they will form the callus tissue.

Organogenesis is the formation or development of an organ or even whole plant by the experimental manipulation of plant cell or tissue in *in vitro* under controlled and aseptic conditions (Hicks, 1980). In most cases, organ production under *in vitro* culture, the factors affecting them and the nature of developmental events favoring the organ determination were clearly understood. Therefore, information from such basic studies was essential for the programming of applied aspects of research works revealed different pattern of organogenesis (Torrey, 1977 and Winton, 1978). This process of organogenesis includes callus formation leading to organ differentiation.

When an explant from differentiated tissue is used for culture on a nutrient medium, the non-dividing, quiescent cells first undergo certain changes to achieve a meristematic state. The phenomenon of the reversion of nature cells to the maristematic state leading to the formation of callus is called ‘differentiation’. The component cells of callus have the ability to form a whole plant, a phenomenon described as ‘redifferentiation’. These two phenomena of differentiation and redifferentiation are inherent in the capacity described as ‘cellular totipotency’, a property found only in plant cells. Although generally a callus phase is involved before the cells can undergo redifferentiation leading to regeneration of a whole plant, yet rarely the differentiated cells can give rise to whole plants directly without an intermediate callus phase. These aspects of tissue culture and its application in ‘clonal propagation’ and in the generation of hereditary variation are called somaclonal variation (Gupta, 1998b).
A rapid and large-scale in vitro clonal propagation system has been developed for *Centella asiatica*, a valuable medicinal herb (Tiwari et al., 2000) and *Kaempferia galanga*, a rare folk medicinal herb (Shirin et al., 2000). Large-scale and unrestricted exploitation of the natural resource to meet its ever increasing demand by the Indian pharmaceutical industry is coupled with limited cultivation and attempts for its replenishment. The wild stock of these medicinally important plant species which have been markedly depleted and now they are listed as threatened species by the International Union for Conservation of Nature and Natural Resources (IUCN) as has been reported by Pandey et al. (1993) and Banu and Handique (2003). Tissue culture techniques can play an important role in the clonal propagation of elite clones and germplasms conservation of *Centella asiatica* (Tiwari et al., 2000).

High frequency of adventitious regeneration from a range of explants including mature and immature embryos (Ozgen et al., 1998) and leaflets, petioles and stem have been reported (Ozcan et al., 1996). Although adventitious shoot regeneration is a basic prerequisite for the application of genetic engineering to crop improvement, the production of plants from apical meristem and axillary bud has proven to be the most genetically applicable and reliable method of in vitro micropropagation (Sancak, 1999).

In many plants there are reports of successful callus induction and plantlets regeneration, for example through leaf explants of *Coleus forskohlii* (Reddy et al., 2001), *Anisomeles indica* (John Britto et al., 2002), *Tylophora indica* (Faisal and Anis, 2003), *Cajanus cajan* (Yadav and Padmaja, 2003), *Curcuma amada* (Prakash et al., 2004), *Cucumis sativus* (Filifecki et al., 2005) and *Nicotiana tobacum* (Murnilawati et al., 2006), node explants of *Tylophora indica* (Faisal and Anis, 2005) and *Gynnema sylvestre* (Gopi and Vatsala, 2006), inflorescence explants of *Hosta sieboldiana* (Saito and Nakano, 2002) and *Zoysia* spp. (Poeaim et al., 2005), internode explants of *Ocimum basilicum* (Sudhakaran and Sivasankari, 2003) and *Pogostemon cablin* (Parida et al., 2005), petiole explants of *Salvia canariensis* (Molina, 2004), root explants of *Sorghum bicolor* (Baskaran et al., 2006), immature embryo explants of *Triticum aestivum* (Talukder et al., 2004), cotyledon explants of *Lycopersicon esculentum* (Bhatia and Ashwath, 2005) shoot tip explants of *Camellia sinensis* (Aoshima, 2005) and anther explants of *Narcissus tazetta* (Chen et al., 2005).
Currently, commercial in vitro propagation was carried out through the callus phase on Dieffenbachia cv., (Shen et al., 2007), Prunus avium (Feeney et al., 2007), Saussurea involucrata (Guo et al., 2007), Echinacea purpurea (Jones et al., 2007), Campanula carpatica (Sriskandarajah et al., 2008), Bacopa monnieri (Debnath, 2008b), Sarcostemma brevistigma (Thomas and Shankar, 2009), Acanthophyllum sordidum (Meratan et al., 2009), Phaseolus vulgaris (Arellano et al., 2009), Ocimum sanctum (Shilpa et al., 2010) and Punica granatum (Kanwar et al., 2010).

An efficient in vitro dedifferentiation and plant regeneration from callus culture, is needed for mass propagation and several in vitro manipulation studies including genetic transformation, where direct regeneration may not be effective (Faisal et al., 2006). The development of reliable tissue culture methods for the production and maintenance of callus and regeneration of plants were imperative for the successful application of tissue culture to crop improvement as was proposed by Muthuramu et al. (2007). An efficient in vitro plant regeneration system is a basic necessity for the culture of callus tissue which provides an important technique which can be preliminary to the regeneration of whole plant and established plantlets either directly or via., callus culture from leaf and node explants has been reported (Rathod et al., 2008).

Tissue culture and in vitro plant regeneration systems may provide an alternative means for mass proliferation and ex situ conservation of endangered plant species. Therefore, present communication describes an efficient protocol for morphogenetic callus induction and plant regeneration through indirect organogenesis from various explants like leaf, petiole, internode, inflorescence rachis, root, apical bud, axillary bud, node and inflorescence bud explants derived callus cultures of Plectranthus barbatus. The plant regeneration system established in this study can be applied to mass propagation and conservation of this valuable medicinal plant species.

4.2. REVIEW OF LITERATURE

Mass clonal propagation through cotyledons callus culture of Acacia auriculiformis was explored by Das et al. (1993). Cotyledon callus tissues were differentiated into shoot primordial on MS medium augmented with BAP 5.0mg/l with 2% (w/v) sucrose. It rooted when placed on modified MS medium with IBA 1.0mg/l or 1.5mg/l and sucrose 1.5% (w/v). The regenerated shoots when placed on MS medium with
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GA₃ at 0.25mg/l and continued to produce roots. Organogenesis of shoots and roots were observed from leaf derived callus in *Vigna radiata* (Patel *et al*., 1991) and cotyledon derived callus in *Vigna unguiculata* (Anand *et al*., 2001). Callus culture were initiated from immature cotyledon explants on MS + B5 medium containing IBA 2.0mg/l and KN 2.0mg/l. Adventitious shoots were obtained on Zeatin (1.0mg/l) fortified medium.

Shoot regeneration depended on explant source, medium composition and light photoperiod (Brown and Thorpe, 1986). L. arginine was effective for shoot multiplication in *Agave fourcroydes* (Robert *et al*., 1987). Arti *et al*. (1994) and Nguyen *et al*. (1998) reported induction of callus on MS medium containing 2,4-D at 2.5mg/l and 2.0mg/l respectively. Elkonin *et al*. (1995) found increased proliferation of embryogenic callus on MS medium containing asperagin and proline at a concentration of 3.0mg/l and maintenance of embryogenic callus for over three months on MS containing 20g/l sucrose and vitamin mixture.

Shoot regeneration through organogenesis was achieved by Lisowska and Wysokinska (2000) culturing cotyledon callus on SH medium containing IAA (0.6µM) and BA (4.4µM) or Zeatin (22.8µM). Wysokinska and Swiatek (1989) demonstrated on *Catalpa bignonioides* from hypocotyls derived calluses for shoot regeneration that BA and Zeatin were more effective than other cytokinins.

Shoot regeneration from stem explants of *Hybanthus enneaspermus* was described by Natarajan *et al*. (1999). Nodular calli were obtained on MS medium containing 2.0mg/l 2,4-D and 0.5mg/l BAP. Shoot buds were induced and elongated on MS basal medium with 5.0mg/l BAP, then shoots were rooted in same media with 2.0mg/l IBA. Kelkar and Krishnamurthy (1998), Kelkar *et al*. (1996) and Jaiswal and Narayana (1985) reported that the 2,4-D alone induced callusing. The shoot proliferation was better in medium containing BAP (5.0mg/l). BAP induced higher frequency of shoot proliferation and maximum of multiple shoots.

Jawahar *et al*. (1997) achieved higher frequency of callus induction with increasing concentration of auxins, the optimal levels being 2.0mg/l of IAA/NAA and 1.0mg/l of BAP/KN among the hypocotyls explant of *Lycopersicon esculentum*. Shoots were induced from callus cultures of these explants on the same medium. The regenerated shoots were
rooted on MS medium containing 2.0mg/l IBA. Gunay and Rao (1978) reported that the optimum plant regeneration was observed on MS medium with IAA 0.5mg/l and BAP 2.0mg/l in hypocotyl and cotyledon explants, whereas Padmanabhan et al. (1973) obtained shoots on MS medium containing 0.5mg/l IAA and 3.0mg/l KN.

A high frequency shoot organogenesis protocol was carried out by Reddy et al. (2001) using leaf derived callus of Coleus forskohlii. Optimal callus was developed from mature leaves on MS medium supplemented with 2.4µM KN alone. Shoots were regenerated from the callus on MS medium supplemented with 4.6µM KN and 0.54µM NAA. The highest rate of shoot multiplication was achieved after six subcultures on similar medium. Regenerated shoots were rooted spontaneously on half strength medium devoid of growth regulators, the in vitro raised plants were established successfully in soil.

Efficient plant regeneration was achieved via., organogenesis from callus culture derived from leaf tissue of Echinacea purpurea (Koroch et al., 2002). Proliferating shoot cultures were obtained when leaf explant was placed on MS medium supplemented with BAP (4.44µM) and NAA (0.054µM) with high shoot regeneration frequencies (100%) and high number of shoots (7.7 shoots/explant). Plantlets were rooted on MS media without plant growth regulators and combination with different concentration of IBA (2.46µM, 4.90µM). Saito and Nakano (2002) established a system for suspension culture and plant regeneration of Hosta sieboldiana. Nodular calluses were induced from more than 20% of scape segments on MS medium containing 1.0mg/l picloram, 30g/l sucrose and 2g/l gellan gum. Upon transfer of calluses to the same medium, stably growing suspension cultures were established after one month. Suspension cell clusters regenerated a large number of adventitious shoots following transfer to MS media containing 0.1mg/l in NAA combination with 1.0 or 5.0mg/l TDZ.

Multiple shoot regeneration from nodal explants of Ocimum sanctum has been achieved by Shahzad and Siddiqui (2000) culturing MS medium supplemented with phytohormons. The addition of 50mg/l ascorbic acid to MS checked the release of phenolic exudates. MS + 2,4-D 2.0mg/l proved the best for the induction of organogenic callus and on subculturing callus on MS + BA 5.0mg/l + NAA 0.2mg/l + Glutamic acid
(50mg/l) multiple shoots were differentiated. Callus induction followed by profuse rhizogenesis was observed on MS + NAA (5.0mg/l) + BA (0.5mg/l). Lateral bud break has been obtained on MS + adenine sulphate 5.0mg/l + IAA 0.5mg/l. The microshoots were rooted well on MS + NAA (1.5 mg/l).

Hazra et al. (2002) reported that shoot regeneration from callus in casein hydrolysate (CH) supplemented medium was achieved from rhizome and immature leaf tissue. The highest rate of regeneration was obtained with BA (26.6µM) as the sole hormone. Shoot proliferation rate increased on half strength MS medium containing BA (8.9µM). Microshoots developed on MS medium containing BA (2.22µM) and GA3 (1.44µM) and finely rooted on MS medium containing IAA (11.42µM). The bulbil explants of Agave arzonica Gentry weber and rhizome and stem explants of Agave fourcroydes callused on MS medium containing 2,4-D (Powers and Backhaus,1989) and B5 medium 2,4-D and BA (Robert et al., 1987).

John Britto et al. (2002) standardized a protocol for in vitro nodular organogenic callus from leaf explant of Anisomeles indica on MS medium containing 9.04µM/l 2,4-D. Adventitious buds formed from callus on MS medium augmented with 13.32µM/l BAP coupled with NAA (2.69µM). Regenerated shoots were rooted on MS medium and fortified with 9.84µM/l IBA. For Hybanthus enneaspermus by Natarajan et al. (1999) and Datura metel by Muthukumar et al. (2000) have reported that 2,4-D alone induced callusing (green, hard and compact) when subcultured on MS medium supplemented with different concentrations of BAP/KN (4.44 - 17.76µM/l) and NAA (2.69µM/l) for regeneration of shoots.

Zambre et al. (2002) developed a in vitro protocol for prolific shoot regeneration from two elite genotypes of Lathyrus sativus L. Vegetative apical and axillary bud explants from greenhouse grown plants were used to induce regeneration competent green nodular callus. Callus was induced on two B5 basal media that contained TDZ in combination with NAA or IAA. Although callus induction was achieved on both media, progression to the formation of green-nodular callus, a distinct morphological feature and prerequisite for regeneration, differed. Shoot elongation was achieved on medium that contained BAP, IAA and coconut water. Regenerated shoots were readily converted with
high efficiency into sexually mature plants either by planting shoots that were induced for rooting on hormone free B5 basal medium or by grafting of non-rooted shoots. A single combination of TDZ and IAA can induce green nodular callus and subsequently a mode of shoot regeneration, regardless of the genotypes in *L. sativus* and *Phaseolus* species, which belong to different tribes in the large Fabaceae family (Zambre et al., 2001 and Dillen et al., 2000).

Callus initiation from the leaf and internodal explants of *Ocimum basilicum* was observed after 15 days from cut faces of the explants inoculated on MS basal media supplemented with 2.0mg/l 2,4-D and 1.0mg/l KN and white friable, compact and nodular calli were observed (Sudhakaran and Sivasankari, 2003) *O. sanctum* showed 2.0mg/l 2,4-D as the best suitable auxin for callus induction (Shahzad and Siddiqui, 2000). Callusing response in *Ocimum* species was observed on MS basal medium with 0.5mg/l 2,4-D and 1.0mg/l KN in *O. viride* and 0.5mg/l BA in *O. kilimandscharicum* x *O. basilium* (fertile hybrid). Kumar and Bhavanandan (1988) achieved good callus mass in *Plumbago rosea* using 2,4-D (2.5mg/l) + KN (1.5mg/l). Sarasan et al. (1994) obtained embryogenic callus in *Hemidesmus indicus* on MS medium supplemented with 2,4-D (1.0mg/l) and KN (1.0mg/l).

*In vitro* callus (Sharma and Wakhlu, 2003) was achieved from petiole explants of *Heracleum candicans* on MS medium fortified with BAP + 2,4-D (0.5mg/l each). Shoot differentiation from callus on MS medium containing 1.0mg/l BAP and 0.2mg/l NAA finely rooted plantlets on MS medium supplemented with 1.0mg/l IBA.

An efficient protocol for plantlet regeneration through the leaf calli has been established by Nagaraja et al. (2003) on *Andographis alata*. Leaf explant proliferated into luxuriant callus mass on MS medium fortified with 7.0µM NAA. Callus was differentiated on MS medium enriched with 2.0µM BAP and 0.5µM NAA then the microshoots rooted well on MS fortified medium with 1.0µM BAP + 1.0µM NAA. Muthukumar et al. (1996) noticed the callogenic influence of interaction of BAP and NAA in Cowpea explant culture. In most of the dicotyledonous species 2,4-D is the principal hormone required for the induction of callus and maintenance of culture (Sunderland, 1973; Britto et al., 1995 and Iyer et al., 1998). The reports of Krueger et al. (1982), Yamamoto and Yamada (1986) and Brission et al. (1988) pointed out that the *in vitro* derived calli act as an alternative to the medicinally useful plant parts for the biosynthesis of active constituents.
Regeneration of multiple shoots via., callus induction and organogenesis was achieved by Huda et al. (2003) using cotyledon explant of *Cicer arietinum*. Highest (95) percentage of callus formation was observed on MS + 3.0mg/l 2,4-D + 3.0mg/l BAP. Callus differentiated on MS medium fortified with 2.0mg/l BAP and 0.5mg/l NAA. The regenerated shoots developed highest (77) percentage of roots on half MS medium containing 1.0mg/l IBA. In auxin (2,4-D) alone or in combination with cytokinins (BAP, KN) 100% callus induction has been reported by Panday and Ganopaty (1984) and Anil et al. (1986). The efficacy callus induction and proliferation studies by Jeyaseelan and Rao (2005) showed that IBA induced the highest callus production (94%) in leaf explant and internode (100%).

Callus induction from petiole explants has been achieved (Thao et al., 2003) in *Alocasia micholitziana*. The highest percentage of callus induction was obtained on MS medium supplemented with 0.5µM 2,4-D and 0.5µM KN. Shoots were regenerated at highest frequency of 33.3% under light condition when 0.5µM BA was added to the MS medium. Shoots were rooted on hormone free MS medium. In general callus induction in monocotyledonous plants requires considerably longtime for its initiation as has been investigated by Geier (1986). Presence of auxins inhibited initiation of shoots from the calluses (Yam et al., 1990). In addition of cytokinins either enhanced the response or changed the direction of the response of the callus (Lin et al., 2000). Cotyledonary leaves from mature embryo callused on MS medium supplemented with NAA 5.0mg/l and KN 5.0mg/l, the calli were differentiated on MS medium containing BA 0.2mg/l. Rooting achieved on WP medium containing IBA 5.0mg/l was reported (Sharma et al., 2003).

Axillary bud multiplication and indirect organogenesis were established by Vadawale et al. (2004) using *Withania somnifera*. MS medium containing with 3.32µM BAP, 1.16µM KN and 0.98µM IBA induced an average of five shoots per node. Callus initiated from the basal cut end explants differentiated into more than 20 shoots on MS medium with 4.43µM BAP and 0.98µM IBA. *In vitro* shoots were rooted on MS medium with 3.69µM IBA.

A protocol was outlined by Prakash et al. (2004) for direct and indirect regeneration through rhizome and leaf sheath explants of *Curcuma amada*. MS medium fortified with 4.44µM BA and 1.08µM NAA was the best concentration for shoot
proliferation on rhizome explants. For indirect regeneration semi friable callus obtained from leaf sheath explants on MS medium with 9.0μM 2,4-D was used and transferred to 8.88μM BA and 2.7μM NAA containing medium produced optimum shoot initiation and plantlets development.

_In vitro_ plantlet regeneration in the main commercial variety of _Litchi chinensis_ was achieved from callus cultures derived from young, tender leaf explants on MS medium. Callogenesis was obtained in all media supplemented with auxin, but most prominent in media supplemented with 2,4-D (1.5mg/l) with or without BAP and KN. Nodular compact callus obtained in the 2,4-D and BAP treatment proliferated and differentiated into shoots. When transferred to MS medium supplemented with BAP (2.0mg/l) and IAA (3.0mg/l), regenerated shoots produced prominent roots when transferred to MS medium supplemented with IBA (2.0mg/l). Somatic embryogenesis was observed when callus growing on MS medium supplemented with 2,4-D (1.5mg/l) was transferred to medium devoid of 2,4-D (Puchooa, 2004).

Nodal explant of _Sesbania drummondii_ when cultured on MS medium containing 22.2μM BA proliferated into multiple shoots. MS medium 2.2μM and 4.5μM thidiazuron induced 5 - 6 shoots per stem node. Callus induced on cotyledonary explants when subcultured on 2.2μM thidiazuron containing medium resulted in its mass proliferation having numerous embryoid like structures. IBA 0.24 - 2.46μM was found suitable for root induction (Cheepala _et al._, 2004). Chauhan and Kothari (2004) reported that callus induction and subsequent plant regeneration from seed obtained MS medium supplemented with 11.31µM 2,4-D and 2.68μM NAA + 8.87μM BAP, respectively.

An efficient regeneration protocol was developed by Thomas and Puthur (2004) on _Kigelia pinnata_ of nodal segments. The highest friable organogenic callus (100%) was observed when nodal segments were cultured on MS medium fortified with 3.0μM 2,4-D. The mophogenic callus maintained high regeneration when calli were subcultured on MS medium containing TDZ (3.0μM) and NAA (0.5μM). The regenerated shoots showed maximum rooting on half strength MS medium fortified with 4.0μM IBA. TDZ, a synthetic phenylurea, is considered to be one of the most active cytokinins for shoot induction in plant tissue culture (Huetteman and Preece, 1993). TDZ induced shoot
regeneration from different explants of many recalcitrant species as well as from medicinal plants (Bhagwat et al., 1996; Seneviratne and Flagmann, 1996; Murthy et al., 1998 and Liu et al., 2003).

Siddiqui et al. (2004) standardized a reproducible protocol for plant regeneration from nodal segments derived callus of *Withania somnifera*. The organogenic callus induction was observed in MS medium containing 1.0mg/l BAP and 2.0mg/l KN. Shoots differentiated best (80%) from node derived callus on MS medium containing 1.0mg/l BAP and 2.5mg/l KN, then regenerated shoots rooted best on MS medium supplemented with IBA and KN (1.0mg/l). Gita and Grover (1999) used KN and with BAP for callus induction with 84% frequency. Significant improvement in shoot formation over control has been achieved with the addition of cytokinins like BAP and KN has been reported (Conchoa et al., 1992 and Le, 1994).

Vila et al. (2004) carried out in vitro regeneration of shoots from leaf explants of *Melia azedarach* L. The leaves of 10 - 15 years old plants of seven genotypes were cultured on MS medium supplemented with 1.0mg/l BAP + 1.0mg/l KN + 3.0mg/l AdS. Shoot regeneration occurred in leaf explants of all (4, 14, 24, J1, J2, LP and R) genotypes tested. The best genotype for shoot regeneration was clone 4. Rooting was induced on MS medium supplemented with 2.5mg/l IBA. In *Morus alba* the shoot formed in the transitional zone between the midrib and petiole (Oka and Ohyana, 1981).

Regeneration of multiple shoots via callus induction and organogenesis were achieved by Rahman et al. (2004b) on *Elaeocarpus robustus*. The best organogenic callus was found on internodal explants on modified MS medium supplemented with 0.5mg/l BA + 0.5mg/l 2,4-D. Adventitious shoot buds occurred when the calli were transferred on modified MS medium fortified with 1.0mg/l BA + 0.1mg/l NAA. Plantlets developed roots when in vitro developed microcuttings were implanted on modified MS medium with 0.2mg/l of IBA. Superior effect of BA – NAA combination on adventitious bud proliferation from leaf explant has been reported by Islam et al. (1992) for *Aegle marmelos*. Organogenesis and plants formation in presence of BA with NAA was reported from cotyledon explants (Bornman, 1983).
The leaf discs and stem segments of *Hypericum perforatum* regenerated from different concentrations of sucrose and phytohormones were tested (Ayan *et al.*, 2005). The segments were cultured on MS medium supplemented with KN and 2,4-D and sucrose concentration (30, 40, 50g/l). The highest value was obtained from MS medium supplemented with 30g/l sucrose, 0.5mg/l 2,4-D and 0.5mg/l KN in term of callus initiation frequency. Calli were subcultured on MS basal medium containing 1.0mg/l BA and for shoot induction, while the same basal medium supplemented with 1.0mg/l IBA was employed for rooting. The highest calli were obtained from stem segment cultured in the medium supplemented with 1.0mg/l 2,4-D and 1.0mg/l KN in *H. perforatum* (Bezo and Stefunova, 2001).

Manoharan *et al.* (2005) established an efficient protocol for induction of embryogenic callus and direct plant regeneration in *Vigna mungo*. Embryogenic callus was achieved in B5 medium + 2,4-D (2.0mg/l) and BAP (0.2mg/l), when leaf cultures were incubated for 45 days without the periodical subculture for 28 days. Direct plant regeneration was achieved from cultured cotyledonary nodes from 3.5 days old seedlings, on B5 + BAP (2.0mg/l) and choline chloride (5.0 µM). The regenerated shoots were rooted in B5 medium + 2,4-D (1.0mg/l) and subsequently hardened.

A protocol of high frequency shoot organogenesis and plant establishment was analyzed by Faisal and Anis (2005) using stem derived callus of *Tylophora indica*. Callus was developed on MS medium fortified with 10.0µM 2,4,5-T. Multiple shoot induction was achieved on MS medium containing 5.0µM KN. The developed shoots rooted best on half strength MS medium supplemented with 0.5µM IBA.

Shoot regeneration from *in vitro* seedlings derived leaf explants of *Hagenia abyssinica* was achieved by Feyissa *et al.* (2005) on MS medium containing lower concentration of TDZ (1.0mg/l), but higher concentrations of promoted callus induction. Around 96 - 100% regeneration was obtained between 1.0 and 10µm TDZ. The average number of shoots per explant at 1.0µm TDZ was 8.4 ± 4.8. An *in vitro* rapid mass propagation of disease free planting material of Patchouli was standardized by Parida *et al.* (2005). Callus were obtained from leaf disc and internode explants on MS basal medium supplemented with NAA 1.5mg/l and BA 0.5 mg/l. High frequency shoot regeneration
was obtained when callus was subcultured on half strength MS basal medium supplemented with BA 1.0mg/l. Complete plantlets were obtained when the calli were subcultured on half strength MS basal medium with BA (0.1mg/l).

Faisal et al. (2005) standardized a protocol for high frequency of plant regeneration from petiole explant of *Tylophora indica* on MS supplemented medium. Maximum of callus induction noticed on media with 2,4-D 10µM + Thidiazuron 2.5µM and adventitious shoots induction 2.5µM TDZ. The elongated shoots were rooted best on half strength MS medium containing 0.5µM IBA. Govindaraju et al. (2003) developed callus induction from internodal segment, leaf, root and petiole explants on MS and B5 medium supplemented with 2,4-D and NAA either alone or in combination with KN.

Among different combinations of the PGRs in the MS medium 0.89µM BAP + 4.52µM 2,4-D and 4.44µM BAP + 5.37µM NAA showed the highest performance to produce callus with the frequency of 90% and 80% respectively on hypocotyl explants of *Phellodendron amurense* (Azad et al., 2005). Rani and Grover (1999) reported that 2.0mg/l 2,4-D and 0.2mg/l KN was best for callusing from hypocotyl, leaf and root explants of *Withania somnifera*. Camper et al. (1997) suggested that BAP with 2,4-D is best for embryogenic callus formation in *Ginkgo biloba*. Choi et al. (1996) reported that LS liquid medium containing 0.44µM BAP and 2.22µM 2,4-D was more effective for callus induction of *P. amurense*. Ikuta et al. (1998) reported that 4.5µM 2,4-D and 0.44µM KN was the best formation for callus induction of *P. amurense* under a dark condition. Hypocotyl derived calli were regenerated on MS medium supplemented with 4.44µM BAP + 5.37µM NAA or 0.22µM BAP + 4.92µM IBA on *P. amurense* has been suggested by Azad et al. (2005).

*In vitro* mass multiplication of *Saussurea obvallata* was established by Dhar and Joshi (2005) using leaf explants. 100% callusing was achieved in MS medium supplemented with 2.5µM BA and 1.0µM NAA and 100% differentiation along with a multiplication rate of 12 shoots per explant with a combination of 5.0µM BA and 1.0µM NAA then *in vitro* rooting of shoots was achieved on half strength MS medium containing 2.5µM IBA. Cytokinin – auxin combination have been widely used for callus regeneration developed for the species of *Saussurea* and other members of Asteraceae family (Knittel
et al., 1991; Chraibi et al., 1992 and Barker et al., 1999). Hu et al. (2005) established callus induction from petiole explant of Amorphophalus rivieri on MS medium containing 5.37µM NAA and 4.44µM BA. The shoot and corm organogenesis occurred from the compact calli when they were transferred to a medium containing 0.54µM NAA and 4.44µM BA.

Gopi et al. (2006) induced callus culture from nodal segments and leaf explants of Gymnema sylvestre on MS medium containing basic salts and 30g/l sucrose supplemented with different concentrations of 2,4-D, NAA, IAA, IBA, KN and BA. Callus induction was observed in 0.5mg/l 2,4-D supplemented medium for both explants. The harvested cell biomass was subjected to extraction of active principles.

An efficient and reproducible protocol has been developed from cells or tissues of agronomically important Indian Sorghum [Sorghum bicolor (L.) Moench]. Callus induction and plant regeneration were achieved on transverse thin cell layers (TCL) of roots on MS medium supplemented with 2,4-D (4.5-18.1µM) and NAA (5.4 - 21.5µM), IAA (5.7 - 22.8µM) and IBA (4.9 - 19.7µM) and combined with 10% (v/v) coconut water (CW) which was used for callus induction. The calli were subcultured on MS supplemented with BAP (2.2 - 17.8µM) combined with 2,4-D (2.3µM) or NAA (2.7µM). High efficient differentiations of multiple shoot buds were initiated within 4 weeks of culture. Roots developed on half MS medium containing 1AA (2.9 - 28.5µM). Rooted plants were successfully acclimatized (Baskaran et al., 2006).

A high frequency shoot organogenesis and plant establishment protocol has been developed for Ruta graveolens from stem derived callus (Faisal et al., 2006). Efficient de novo organogenic callus medicated shoot bud differentiation was developed from stem explants on MS medium composed of 2.5µM BA + 10µM 2,4-D. The maximum number of (98.2 ± 1.8) shoots per callus clump was observed on MS medium supplemented with 7.5µM BA in combination with 2.5µM NAA. In vitro rootings were obtained on half salt MS medium supplied with IBA on which 90% of cultured shoots produced healthy roots. Rooted shoots, following acclimatization in green house, were successfully transferred to soil with 90% survival.
An *in vitro* protocol for plantlets regeneration from cotyledon and shoot tip explants from 10 days old *in vitro* grown seedlings of *Pseudarthria viscida* was developed (Deepa et al., 2006). Cotyledon and shoot tip explants induced profuse callusing on MS medium supplemented with 2,4-D (1.5 - 2.0mg/l) and BAP (1.0 - 1.5mg/l). Shoot regeneration was achieved on both half and full strength MS medium supplemented with BAP (2.0mg/l) and shoot elongation, when GA$_3$ (1.0mg/l) was added, shoots were rooted on half strength MS medium fortified with IBA (1.0mg/l). Plantlets were successfully hardened.

Micropropagation protocol was developed for *Embelia ribes* using inflorescence explants (Shankarmurthy and Krishna, 2006). The inflorescence segments proliferated into luxuriant mass of callus on MS medium supplemented with IBA (3.5mg/l) and KN (0.5mg/l). The combination of KN and NAA on the range of 3.0 and 0.4mg/l, respectively provoked the calli to differentiate into both shoots and roots initials from the callus. The regenerants were transferred to the pots containing sterilized soil and hardened for a week.

Moghaieb et al. (2006) developed a protocol for plant regeneration from hypocotyls explant derived embryogenic callus culture. Embryogenic calli were formed within two weeks on MS medium augmented with 1.0mg/l 2,4-D, then adventitious shoots emerged from the embryogenic callus in the presence of 4.5mg/l BA. Narasimhulu and Chopra (1988) effected shoot production efficiency and became higher when the medium was supplemented with higher concentration of BA (2.0 - 4.0mg/l). Muhammad et al. (2002) reported that the highest frequency of plant regeneration was achieved on the medium with 2.0mg/l BA and 0.5mg/l IAA using hypocotyls derived calli.

Pareek and Kothari (2007) standardized protoplast isolation, culture and plant regeneration from leaf mesophyll tissue of *Dianthus caryophyllus*. The enzyme mixture containing 2% cellulase and 0.1% pectolyase was found most suitable for high yield and viability of protoplasts. Protoplasts cultured in MS liquid medium supplemented with BAP (1.0mg/l), 2,4-D (2.0mg/l), NAA (2.0mg/l) and mannitol (9%). Microcolonies were formed after four weeks of culture in the dark at 26 ± 1°C. Microcolonies were subcultured on similar medium for development of callus masses. After 2 weeks, callus gave rise to green shoots on GA$_3$ (0.5mg/l) supplemented medium. The shoots were rooted on medium supplemented with IBA (2.0mg/l) and were successfully transplanted to field conditions.
A protocol was standardized for bud proliferation and direct organogenesis for three hybrid Tea varieties of Rose *viz.*, Christion dior, Papa meilland and Black lady (Chavan et al., 2007). MS medium with 0.18µM BAP and 0.25µM KN induced an average of four shoots per node. The AdS (3.0mg/l) was added to all media in order to increase multiplication rate. Callus was obtained from basal cut end explants, when cultured on MS medium with 2.0µM IBA. Callus differentiated into more than seven shoots on MS medium with 0.72µM BAP, 0.36µM KN and 10% CW. Shoots were rooted after pulse treatment with 2.0µM IBA for 48 hrs and then transferred to MS medium containing 0.18µM BAP and 0.24µM KN. 90% of the rooted shoots survived when transferred to green house and subsequently to the field.

Chitradevi and Kamalam (2007) established *in vitro* mass propagation for IUCN red listed medicinal plant *Nilgirianthus ciliatus*. Leaf explants were found to be best suited for callus induction and subsequent organogenesis on MS medium with 0.5mg/l IAA and 5.0mg/l BA. Maximum shoot regeneration from internodal callus was obtained on medium with 0.5mg/l IAA and 5.0mg/l BA. Direct shoot regeneration from nodal explants was optimum on the medium containing 1.0mg/l IAA and 5.0mg/l IBA. *In vitro* shoots were rooted on MS medium supplemented with 0.5mg/l IBA and 3.0mg/l IAA.

A novel protocol for indirect shoot organogenesis of *Dieffenbachia* cv. Camouflage was established by Shen *et al.* (2007) using leaf explants excised from *in vitro* shoot cultures. The frequency of callus formation reached 96% for explants cultured on MS basal medium supplemented with 5.0µM TDZ and 1.0µM 2,4-D. The number of shoots regenerated was high, with up to 7.9 shoots produced per callus cultured on basal medium supplemented with 40µM N6-(D2-isopenteny) adenine and 2.0µM IAA. Regenerated shoots rooted well in a soil-less substrate, acclimatized *ex vitro* at 100%, and grew vigorously under shaded greenhouse conditions. Voyiatzi and Voyiatzis (1989) found that 2iP was more effective in inducing lateral shoot multiplication in *Dieffenbachia* than kinetin, and 80µM 2iP with 2.0µM IAA was optimal for shoot formation of *Dieffenbachia exotica* cv. Marianna.

Sharma *et al.* (2007) established a protocol for *in vitro* plant regeneration from cotyledon and hypocotyl explants of bell pepper (*Capsicum annuum* L. cv.). Morphogenetic responses were obtained from bell pepper of cotyledon and hypocotyl
segments were cultured on MS medium with various plant growth regulators. Highest shoot regeneration (44.40%) was obtained on MS medium containing 6.0mg/l BAP + 0.3mg/l IAA from cotyledon explants. In hypocotyl explants only callus formation and root regeneration was observed. The elongated shoots were transferred to root regeneration media containing IAA (0.1 - 0.5mg/l) and NAA (0.1 - 0.5mg/l).

Mishra et al. (2007) reported that an optimal in vitro propagation procedure was achieved using shoot cultures of Gmelina arborea by Silver nitrate. The proliferated axillary shoots were regularly subcultured on MS medium with 1.5mg/l BA. To reduce callus formation and improve shoot multiplication rate, explants were implanted on MS medium supplemented with 1.5mg/l BA + range of concentration (0 - 4.0mg/l) silver nitrate. The culture grown without silver nitrate developed huge callus at cut end with very poor multiplication rate. Silver nitrate 4.0mg/l significantly reduced callus formation and facilitated enhanced multiplication rate on MS medium supplemented with 1.5mg/l BA.

Smila et al. (2007) successfully micropropagated by culturing nodal and internodal segments of Passiflora foetida L. Maximum percentage of shoot proliferation was achieved, when cultured on MS basal medium fortified with BAP (4.44µM) and callus formation was achieved on MS medium augmented with 2,4-D (4.52µM). Maximum percentage of shoot proliferation from the internodal derived calli was achieved on MS medium supplemented with BAP (2.22µM) and (4.44µM). The in vitro raised shootlets were rooted on MS medium fortified with IBA (4.90µM).

Morphogenetic responses of callus cultures of Cucumis sativus on MS medium supplemented with 2.5mg/l BAP + 0.5mg/l NAA had best shoot induction (Mustafa and Ballaiah, 2007). In vitro studies were carried out by Muthuramu et al. (2007) using various explants through callus induction and efficient plant regeneration in Pearl millet. MS medium augmented with 2.0mg/l 2,4-D induced maximum callus formation. MS medium supplemented with 2.0mg/l BAP + 0.5mg/l IAA for shoot formation and 1.0mg/l IBA + 1.5mg/l IAA for root formation were superior to other hormonal combinations in terms of regeneration.

The effects of explant types, pre treatments, basal media, and phloroglucinol on cultivars Bing, Sweetheart, and Lapins were investigated (Feeney et al., 2007) using Prunus avium. Callus developed on four explant types: apical shoot tips isolated from
orchard trees; and punctured shoot tips, stem sections, and shoot bases of *in vitro* shoot cultures. Callus formed on Bing (5%), Sweetheart (8%) and Lapins (20%) shoot tips from orchard trees after 4 months on half strength MS medium with 3.0µM BA. *In vitro* derived explants formed callus after 3 months on WP medium with 3.0µM BA punctured shoot tips (Sweetheart and Lapins 67%), stem sections (Sweetheart 31%, Lapins 27%), and shoot bases (Sweetheart 10%, Lapins 17%). Pre treatment of shoot cultures on MS with 3.0µM BA and 1.0µM phloroglucinol increased callus formation three folds on shoot base explants. Callus was separated from parental explants and maintained on MS with 3.0µM BA. Shooting was induced by transferring callus to 3.0µM. At 2 weeks, shoot development approached 100%. Callus and adventitious shoot formation were first observed on necrotic explants isolated from orchard trees. Based on these observations, it was speculated that the process of isolating and sterilizing buds caused wounding, which stimulated callus growth and adventitious shoot regeneration (Bhatia *et al.* 2005). Oh *et al.* (1991) evaluated the effect of MS and half strength MS basal media on organogenic callus formation on shoot tip explants and concluded that the lower salt medium whereas either 4.44µM BA (25%) or 0.44µM BA (30%) was best for callusing. Matsuta and Yamaki (1988) achieved even greater callus growth on leaf disks after 35 days on MS supplemented with BA and 2,4-D or NAA, there was an increase of more than 50 times the initial mass using sweet cherry cultivars. Matt and Jehle (2005) described additional combinations of low macronutrient containing basal media for shoot regeneration in leaf and internode sections.

Jones *et al.* (2007) investigated thidiazuron (TDZ) induced morphogenesis of *Echinacea purpurea* L. and to assess the possibility of developing a liquid based protocol for rapid micropropagation. Callus development and root organogenesis were observed on leaf explants cultured on media containing 2,4-D or dicamba, but no plantlets were regenerated. The highest rate of regeneration was observed for explants cultured on medium with TDZ at 2.5µM or higher. Tissue derived from 1.0µM TDZ treatments was used to initiate liquid cultures. All liquid treatments produced a similar number of regenerants but significantly more healthy plants were obtained from cultures grown in the presence of 0.1 and 1.0µM TDZ. Mithila *et al.* (2003) also reported shoot organogenesis at lower concentrations of TDZ and somatic embryogenesis in higher concentrations in leaf explant cultures of African violet (*Saintpaulia ionantha*).
Chapter – IV Indirect Organogenesis

An *in vitro* protocol was developed for rapid multiplication of plantlets from leaf explants of *Cornukaempferia larsenii*. The highest number of callus formation were obtained from young leaves cultured on MS medium fortified with 0.5mg/l 2,4-D in the light condition. The callus could be regenerated to plantlets in media added with various concentrations of NAA and BA (Saensouk *et al.*, 2007). *Zantedeschia aethiopica* calli cultured on MS medium supplemented with IAA 1.5mg/l and KN 1.5mg/l for maximum number of plantlets was produced by Maurya and Anand (2007).

Micropropagation was attempted using leaf explants of *Enicostemma littorale* on MS medium. Maximum callus was obtained with 1.0mg/l of each KN and 2,4-D. When callus was subcultured on medium supplemented with IBA (2.0mg/l) and BAP (4.0mg/l) root initiation, callus proliferation as well as maximum number of multiple shoots were regenerated at a time (Rathod *et al.*, 2008).

A reproducible protocol for *in vitro* propagation of ashwagantha through leaf and nodal segments was achieved by Madhavilatha and Singh (2008). Axillary leaves gave better callus formation on MS + 2,4-D (2.0mg/l) and KN (1.0mg/l). Shoots differentiated best from leaf calli on MS medium supplemented with BAP (2.0mg/l) and IAA (0.5mg/l) and nodal segment on MS medium containing BAP (2.0mg/l) and IAA (1.0mg/l). Shoots were rooted on MS half strength containing 2.0mg/l IBA.

Reddy and Naidu (2008) evolved a reproducible protocol for *Azadirachta indica* through leaf explants. Callus was initiated from young leaves on MS medium supplemented with BA (2.0mg/l) and 2,4-D (0.5mg/l). Organogenesis was achieved by transferring callus on MS medium containing TDZ (3.0mg/l) and adenine (1.0mg/l) getting a very high frequency of shoot regeneration 81.3%. Well grown shoots rooted easily on half strength MS medium supplemented with IBA (2.0mg/l) + IAA (0.5mg/l) and 80 - 90% rooting was achieved. Plantlets were hardened and established very well in the nursery.

An efficient micropropagation protocol based on multiple shoot induction and callus regeneration has been standardized by Thomas and Shankar (2009) in *Sarcostemma brevistigma*. The nodal cuttings were cultured on MS medium supplemented with BA (0.5 - 8.0µM) or KN (0.5 - 8µM) alone or in combination with NAA (0.5 - 1.5µM).
Maximum multiple shoot induction was observed on MS medium supplemented with 4.0µM BA. On this medium, 100% cultures responded with an average number of 11.3 shoots per explant. The addition of 1.0µM NAA along with 4.0µM BA gave rise to an average number of 10.9 shoots with an average shoot length of 1.8 cm. Luxuriantly growing callus was obtained on MS medium supplemented with BA (5.0µM) and 2,4-D (2.0µM). The callus was subcultured on MS medium supplemented with BA (2.0 - 15µM) or KN (2.0 - 15µM) alone or in combination with NAA (0.5 - 2.0µM) for shoot organogenesis. Optimum callus regeneration was obtained on MS medium supplemented with 10µM BA and 1.0µM NAA. The shoots were rooted on half strength MS medium supplemented with NAA (1.0 - 7.0µM) or IBA (1.0 - 7.0µM). BA induced multiple shoot induction has been reported in *Terminalia bellirica* (Ramesh et al., 2005), *Quercus euboica* (Kartsonas and Papafotiou, 2007), *Eclipta alba* (Dhaka and Kothari, 2005) and *Ulmus* (Thakur and Karnosky, 2007). Callus induction through an auxin cytokinin combination has been reported for several systems, including *Valeriana edulis* (Castillo et al., 2000), *Lathyrus sativus* (Zambre et al., 2002), *Dieffenbachia* (Shen et al., 2007). Multiple shoot induction, callus organogenesis and somatic embryogenesis has been reported in *Hemidesmus indicus* (Sreekumar et al., 2000), *Holostemma* (Sudha et al., 2000; Martin, 2002) and *Tylophora indica* (Thomas and Philip, 2005 and Thomas, 2006).

Meratan et al. (2009) studied the effect of various hormonal combinations on callus formation and regeneration of shoot and root from leaf derived callus of *Acanthophyllum sordidum*. Calli were induced from leaf explants on MS medium containing 4.52µM 2,4-D + 4.65µM KN. Maximum growth of calli and the most efficient regeneration of shoots and roots occurred with 2.69µM NAA, 2.69µM NAA + 4.54µM TDZ and 2.46µM IBA.

A protocol for *in vitro* regeneration via, indirect organogenesis for *Phaseolus vulgaris* was established by Arellano et al. (2009). The best callus production was obtained in medium containing 1.5µM 2,4-D. After 2 weeks of growth calli were transferred to shooting medium containing 22.2µM BAP. Shoots regenerated with a frequency of approximately 0.5 shoots per callus, and upon transfer to rooting medium these shoots produced roots with 100% efficiency.
Kanwar et al. (2010) reported that in vitro germinated seedlings explants of cotyledon of *Punica granatum* L. were incubated on solid MS medium supplemented with 21µM NAA and 9.0µM BA, 80% of explants developed callus. A high frequency of shoot organogenesis were obtained when explants were incubated on MS medium supplemented with 8.0µM BA, 6.0µM NAA, and 6.0µM GA₃. However, adding 24µM silver nitrate (AgNO₃) to this medium markedly enhanced shoot regeneration frequency (63%) and mean number of shoots per explant (11.26) and length of shoots (2.22 cm). Highest frequency of *in vitro* rooting, mean number of roots/shoot (4.32), and mean root length (2.71 cm) were obtained when regenerated shoots were transferred to half strength MS medium supplemented with 0.02% activated charcoal.

Shilpa et al. (2010) cultured young leaf explants of *Ocimum sanctum* on solidified MS medium supplemented with 2.0mg/l NAA and 0.2mg/l KN developed rhizogenic callus. When these were subcultured onto MS medium supplemented with 1.5mg/l 2,4-D and 0.5mg/l NAA, friable rhizogenic callus were observed. Upon transfer of this friable callus onto liquid MS medium containing 4.0mg/l NAA and 1.3mg/l BAP. Irvani et al. (2010) employed for the large-scale multiplication and conservation of germplasm on *Dorema ammoniacum* a native medicinal plant in Iran, is classified as a vulnerable species. Various explants were cultured on the medium, the best callus responses were observed from root segments on MS medium containing 1.0mg/l NAA and 2.0mg/l BA. The calli were subcultured on MS medium supplemented with 2.0mg/l BA and 0.2mg/l IBA produced the highest frequency of shoot regeneration (87.3%) in hypocotyl-derived callus. The optimal medium for rooting contained 2.5mg/l IBA on which 87.03% of the regenerated shoots developed roots.

### 4.3. MATERIALS AND METHODS

#### 4.3.1. Source of Explants

*In vivo* and *in vitro* plants of *Plectranthus barbatus* were used as the source of explants. The first fully expanded and second leaf over 1.0 cm long from apical buds and petiole, internode and root explants were made into 0.5 - 0.8 cm long segments and inflorescence rachis, inflorescence flower bud, node, apical bud and axillary bud were cut into 0.6 - 0.8 cm long and the segments were cut with a sharp sterile blade and collected for the present investigation.
4.3.2. Sterilization of Explants

All the explants from young plant were excised with sterile blade and collected in a beaker. The excised explants were thoroughly washed with running tap water for 10 to 15 minutes. Thereafter, the explants 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 for five times.

4.3.3. Inoculation of Explants

The explants were inoculated in a laminar air flow hood and laminar air flow chamber was sterilized with 70% ethanol and by UV-irradiation for 15 minutes. The leaf explants were inoculated in such a way that either the adaxial or abaxial surface was touching the agar stands of culture tubes and petiole, internode, root and inflorescence rachis were placed both in vertical or horizontal position, while inflorescence bud, node, apical bud and axillary bud explants were cultured in 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 a 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.

4.3.4. Culture Conditions

The cultures were maintained at 25 ± 2°C under 16/8 hrs light/dark conditions of 80µEms-2s-1 irradiance provided by fluorescent lamps (TL 40W/54 cool-day light) for callus induction and plantlets regeneration.

4.3.5. Callus Induction

Different explants were cultured on MS basal medium containing 3% (w/v) sucrose, 0.8% (w/v) agar and various concentrations of auxins (2,4-D, NAA and IAA) alone or in combination with cytokinins (BAP, KN, TDZ, AdS and CW) for optimal callus induction. In the present investigation, data were recorded at 20 and 30 days after inoculation of explants on the regeneration medium. The calli were graded according to
their colour in a symbol of B - Brown; G - Green; W - White; LG - Light green; GY - Gleenish yellow; GW - Greenish white; GB - Greenish brown; LB - Light brown; WB - White brown. The nature of callus was measured by the callus compactness and graded into three categories: compact (C), less compact (LC) and friable (F). Abundance of callus was measured by a transparent measuring ruler and graded according to their length scale: large (L) = 20 mm and above, medium (M) = 10 to 20 mm and small (S) = 10 mm below. The calli were subcultured and maintained in in vitro conditions. The effects of these quantitative characters with duration of the time for regeneration were estimated in percentage.

4.3.6. Shoot bud Regeneration and Multiplication

Well developed calli were transferred to regeneration medium containing MS basal medium 3% (w/v) sucrose, 0.8% (w/v) gel and different concentrations of cytokinins like BAP, KN, and TDZ (1.0, 1.5, 2.0, 2.5, 3.0 and 3.5mg/l) in combination with various concentrations of auxins like NAA, IAA, and 2,4-D which were used for shoot buds differentiation. After 4 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. After addition of activated charcoal (0.5 - 3.0%) and coconut water (10 - 15%), there were maximum number of shoots without microbial contamination. The percentage of shoots and plantlets were estimated on the basis of the number of calli.

4.3.7. Shoot Elongation

Proliferated multiple shoots were divided into small clusters of 2 - 3 shoots. They were subcultured on shoot elongation medium containing GA3 (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 16/8 hrs light/dark photoperiod. After two weeks, shoots longer than 3.0 cm were counted and transferred to rooting medium.

4.3.8. Root Induction and Transplantation

The longer shoots (3 cm length) were excised and transferred to MS basal medium containing 3% (w/v) sucrose, 0.8% (w/v) agar and different concentrations of IBA, IAA and NAA (0.1 - 2.5mg/l) alone or in combination with cytokinins such as KN and BAP
(0.1 - 2.5mg/l) for root induction. Rooting was observed from two to three weeks. 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 red garden soil, river sand and saw dust in the ratio of 1:1:1 in 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 for two weeks.

4.3.9. Acclimatization and Hardening of Regenerants

As described in chapter - 2

4.3.10. Statistical Analysis

4.3.10.1. 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 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 frequencies of callus induction, shoot bud differentiation and multiplication, number of shoots, shoot elongation, root induction and number of roots/culture were subjected to standard deviation. Mean separation was conducted by using Duncan’s new Multiple Range Test (DMRT) and means were compared with P < 0.05 at level of significance.

4.3.10.2. Calculation

The experimental results were calculated as follows:

<table>
<thead>
<tr>
<th>Frequency</th>
<th>Calculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency of callus induction</td>
<td>No. of explants responded</td>
</tr>
<tr>
<td></td>
<td>--------------------------------------------</td>
</tr>
<tr>
<td></td>
<td>No. of explants cultured</td>
</tr>
<tr>
<td></td>
<td>No. of callus regenerated</td>
</tr>
<tr>
<td>Frequency of shoot bud regeneration</td>
<td>--------------------------------------------</td>
</tr>
<tr>
<td></td>
<td>No. of callus cultured</td>
</tr>
<tr>
<td></td>
<td>No. of shoots rooted</td>
</tr>
<tr>
<td>Frequency of root induction</td>
<td>No. of shoots rooted</td>
</tr>
<tr>
<td></td>
<td>--------------------------------------------</td>
</tr>
</tbody>
</table>
4.4. RESULTS

The first objective in the establishment of indirect *in vitro* regeneration protocol for *Plectranthus barbatus* was to develop an optimal medium for induction of morphogenetic responses. The morphogenetic responses through callus phase of different explants like leaf, petiole, internode, inflorescence rachis, root, apical bud, axillary bud, node and inflorescence flower bud to different levels of auxins and cytokinins combination in Murashige and Skoog medium are listed in Table - 4.1 and Table - 4.7. The highest percentage of culture response and optimal number of shoots from different explants of indirect organogenesis is given in Fig. – 4.1.

4.4.1. Callus Initiation for Morphogenesis

Different types of (leaf, petiole, internode, inflorescence rachis and root) explants produced morphologically different callus on MS medium containing auxins and cytokinins. The plant growth regulators (PGR) combinations and concentrations screened were totally effective for inducing callus formation. The basal medium devoid of any plant growth regulators failed to induce callus in any of the cultured explants. Developmental stage of the explants is important in *P. barbatus* for callus initiation. Based on the preliminary experiments with different stages, good callus initiation was noticed at the end of the week. Assessment of callusing behaviour was made in respect of colour, nature and size of callus at 10, 20, and 30 days of inoculation (Plate - 4.1 and Plate - 4.2). Following main steps in regeneration of plants through indirect organogenesis is given in Fig. – 4.2.

4.4.1.1. Effect of Auxin

a. Effect of 2,4-D

Different concentrations of 2,4-D at the range of 0.5 – 3.0mg/l were used on MS medium. The frequency of callus induction, types of callus and size of callus were influenced by the explants. In all the explants, callus initiation was seen within 10 days of inoculation. Callus initiation was observed at the cut ends of the explants. Maximum induction of callus occurred at 2.0mg/l from leaf and internode explants. The callus of leaf explant was greenish brown and friable, but light brown and friable in case of internode explant. 1.5mg/l 2,4-D was effective and produced greenish white and less compact callus in petiole, light green and less compact callus in the case of inflorescence rachis. In the root explant there was induction of light brown or whitish brown and friable calli. On
increasing concentrations of 2,4-D from 0.5 to 2.0 mg/l, a gradual increase in size of callus was noticed and on further increase of concentration there was gradual decrease in the callus size. At high concentration of 3.0 mg/l there was nil response in petiole, inflorescence rachis and root explants (Table - 4.1).

b. Effect of NAA

Among the different concentrations of NAA tested, 2.0 mg/l effective for callus growth in all the explants except leaf explant produced maximum light green and compact callus at 2.5 mg/l NAA. Within 10 days of incubation all the explants were involved in callus formation. In initial stage inoculums became enlarged, showed curling and later veins and mid-rib became swollen. Sprouting of callus was first initiated from the mid-veins and form the margin of the explant. At low concentration, compact and green or light green in leaf and petiole, greenish yellow colour in internode and inflorescence rachis and less compact and greenish white coloured calli in root explant was seen whereas at high concentration less compact and greenish brown colour in leaf, petiole and internode, light green in inflorescence rachis and calli were friable and whitish brown in colour in root explant were seen (Table - 4.1).

c. Effect of IAA

Among the different concentrations of IAA used, IAA at 2.0 mg/l induced optimum calli in all the explants. Leaf explant produced compact calli while other explants produced less compact calli. Light green calli are favored in leaf, internode and inflorescence rachis whereas greenish brown in petiole and light brown in root explant. As the concentrations of IAA increased, the callus induction frequency decreased except in leaf and petiole explants where IAA had tremendous effect in inducing callus.

4.4.1.2. Effect of auxin with cytokinin

When the MS basal medium supplemented with auxin alone the explant produced callus, but the growth of callus was less and frequency of callusing was low than in combination. Therefore, a low concentration of cytokinin with auxin was added to increase the frequency of callusing.

a. Effect of 2,4-D with BAP

Of the 2,4-D with BAP evaluated for their effect of callus initiation, after 20 days of inoculation the calli became light brown to brown and less compact in colour in leaf
and internode explants. The highest callus induction efficiency occurred with leaf and internode at higher concentration of 2,4-D (2.0mg/l) and low concentration of BAP (0.8mg/l). Petiole explant produced less compact, greenish white callus and inflorescence rachis produced less compact with greenish yellow calli in contrast, root explant produced friable and light brown calli at 2.0mg/l 2,4-D with 0.8mg/l BAP. In root explant presence of 2,4-D (2.0mg/l) with BAP (0.2 - 1.0mg/l) produced most of the calli and they were friable and light brown only. In the media supplemented with 2,4-D (2.0mg/l) and above 1.2mg/l BAP the callogenic response was nil (Table - 4.1).

b. Effect of 2,4-D with KN

Various explants cultured on MS medium containing 2,4-D (2.0mg/l) with KN (0.2 - 1.2mg/l) callused. After 10 days calli were randomly transferred to MS medium supplemented with different concentrations of KN with 2,4-D for their callusing behaviour. Leaf, internode and inflorescence rachis explants produced maximum frequency at 2.0mg/l 2,4-D and 0.6, 0.8mg/l KN, the nature of calli was less compact and light green, light brown and greenish yellow, respectively. Petiole and root explants produced moderate frequency at 2,4-D, 2.0mg/l with KN 0.6mg/l. The nature of callus light brown and friable. Above or below the optimum level of KN (0.6mg/l), the growth rate was decreased. On the MS medium supplemented with 2,4-D (2.0mg/l) with KN (1.0 and 1.2mg/l) callogenic response was nil, mostly the root calli was brownish and friable (Table - 4.1).

c. Effect of NAA with BAP

After 2 weeks of culture, explants on media containing different concentrations of BAP with NAA were observed to form calli. The best callus formation occurred on medium enriched with 2.0mg/l NAA and 0.8mg/l BAP in leaf, internode and inflorescence rachis. The callus was greenish yellow and compact in leaf, light green and less compact in internode and greenish brown and less compact in inflorescence rachis. Petiole explant produced moderate light green and less compact calli and root explant showed small calli light brown and friable or less compact. When the BAP concentration was increased the nature of callus became compact in all the five explants. Low concentration of BAP (0.2mg/l) with NAA produced friable and greenish brown, light brown and white coloured calli from petiole, inflorescence rachis and root whereas compact and less compact with light green calli from leaf and internode, respectively (Table - 4.1).
d. Effect of NAA with KN

Different explants showed differential response in terms of callus formation. Leaf, petiole and internode explants were superior to other explants. Maximum callus formation was observed from these explants on MS Medium containing NAA 2.0mg/l with KN 0.6 or 0.8mg/l, callus induction was observed on the cut surface of the explants within two weeks. Out of five (leaf, petiole, internode, inflorescence rachis and root), the root explants produced friable and whitish brown calli others were less compact in contrast. Their colour differed as greenish brown, light brown, light green and greenish yellow as noticed in leaf, petiole, internode and inflorescence rachis explants, respectively. At low concentrations of KN 0.2mg/l with NAA showed green or light green with less compact or compact calli whereas higher concentration of KN (1.2mg/l) turned brown and produced less compact or friable calli. No signs of callogenic response were observed at KN (1.2mg/l) in root explant (Table - 4.1).

e. Effect of NAA with AdS

AdS with NAA generally induced optimum callusing with all the explants. At 2.0mg/l NAA with 0.2mg/l AdS induced greenish brown, light brown in leaf and petiole, greenish yellow and light green in internode and inflorescence rachis with less compact calli whereas root showed light brown and friable calli. As the AdS concentration increased in the case of NAA, compactness of the calli turned friable. At lower concentration, AdS with NAA induced light green and compact calli and in contrast, higher concentration produced friable and brownish colored calli. Higher concentration of AdS (1.2mg/l) with NAA (2.0mg/l) had ineffective callogenic response in inflorescence rachis and root explants.

f. Effect of NAA with TDZ

Callus induction of high frequency was seen from leaf, internode and inflorescence rachis followed by petiole and root explants on MS medium fortified with lower concentration of TDZ (0.6mg/l) and higher concentration of NAA (2.0mg/l). Root explants produced only friable and brownish or whitish colored calli that did not respond to higher concentration of these combinations. Other explants produced highly compact and light green colored calli at lower concentration of TDZ (0.2mg/l) with NAA (2.0mg/l) (Table - 4.1). As the TDZ concentration increased there was gradual increase in callusing response up to optimum level and on further increase of concentration of TDZ the calli gradually decreased and turned brown and friable.
g. Effect of IAA with BAP

When IAA (2.0mg/l) with different concentrations of BAP (0.2 – 1.2mg/l) were tested, each induced green or light green and less compact or highly compact callus in all the explants except root. Root showed friable light brown callus but in higher concentration of BAP (1.2mg/l) there was no callus induction (Table - 4.1). The frequency of callogenetic responses was more at the concentration of 2.0mg/l NAA and 0.8mg/l BAP from leaf, internode and inflorescence rachis than in petiole. The calli was highly compact and green colored.

h. Effect of IAA with KN

Callus was initiated from the explants on basal medium supplemented with different concentrations of IAA. The IAA at 2.0mg/l with KN (0.2 – 1.2mg/l) had enhanced proliferation of callus efficiency than IAA alone. The maximum callus growth was found with IAA 2.0mg/l + KN 0.6mg/l from leaf, internode and inflorescence rachis explants whereas petiole and root explants showed moderate callogenetic response. Lower concentration of KN supplemented medium produced green and high compact calli among all the explants except root which showed less compact whitish brown coloured calli. Higher concentration of IAA with KN supplemented medium, produced less compact or friable and brown callus (Table - 4.1).

i. Effect of CW with 2,4-D

Depending upon the medium composition and explants, callusing was initiated between 10 – 20 days after inoculation although among the range of concentrations and combination of growth regulators tested, the best callusing was observed on leaf, internode and inflorescence rachis explants cultured on MS medium supplemented with 2,4-D 1.5mg/l + CW 150ml/l, however petiole and root explants produced moderate callusing. The callus was light brown or brown in all concentrations of 2,4-D with CW. Lower concentrations of 2,4-D produced less compact calli, but on increasing the concentration of 2,4-D the callus compactness turned into friable. Low frequency or no signs of callogenesis was observed in the presence of higher concentrations of 2,4-D (2.5 and 3.0mg/l) with CW (Table - 4.1).

j. Effect of CW with NAA

NAA at lower concentration with CW (150ml/l) induced light brown and friable callus from root, while at higher concentration the callus was less compact and small in
size and seen after 20 days of inoculation. Explants at lower concentration of NAA with CW produced compact or less compact and greenish yellow or light green callus. However, the increased concentration of NAA up to 1.5mg/l produced increased callus and a further increase of concentration of NAA resulted in a decline in callogenic response (Table - 4.1).

4.4.2. Regeneration

A plant regeneration system via., indirect shoot organogenesis was established in this study. The type, concentration and combination of plant growth regulators are the key factors for influencing indirect organogenesis in *Plectranthus barbatus*. The different morphological callus growth and regeneration by cytokinins (BAP, KN and TDZ) alone or in combination with auxin (NAA) resulted in different modes of action from selected plant growth regulators.

4.4.2.1. Regeneration of plant from Leaf derived callus

A much reduced number of shoots or no signs of shoots were developed from leaf derived calli cultured on MS basal medium. When the leaf derived calli were subcultured on organogenic medium containing BAP, KN ad TDZ (1.0 - 3.5mg/l), they showed less compact, light green and nodular calli. Among various growth regulators and different concentrations tested, the higher shoot regeneration frequency (80%) and highest number of shoots (21.8 ± 2.91) were recorded at 2.5mg/l BAP concentration. The callus when subcultured onto the MS medium containing different concentrations of KN and TDZ alone produced the highest regeneration frequency (77.1 and 71.4%) and number of (20.9 ± 2.78 and 18.9 ± 2.32) shoots per callus clump, respectively at a concentration of 2.0mg/l KN and TDZ alone (Table - 4.2). Increasing concentration of both PGR produced more shoots at optimum level of concentration and further increase in concentration declined shoot number gradually. The addition of NAA with optimal concentration of BAP or KN significantly increased the frequency of shoot formation. Maximum frequency of regeneration (88.6%) with 28.2 shoots per callus clump was obtained at 2.5mg/l BAP + 0.6mg/l NAA. The KN (2.0mg/l) + NAA (0.6mg/l) showed maximum regeneration frequency (82.9%) with 24.2 shoots per callus clump. Increasing concentration of NAA with BAP or KN possessed organogenic potential with low frequency of regeneration (Plate - 4.3).
4.4.2.2. Regeneration of plant from Petiole derived callus

After two weeks of subculture the petiole derived calli in MS medium containing different concentrations of BAP, KN and TDZ were observed. The optimum shoot formation occurred on MS medium enriched with BAP 2.5mg/l and maximum frequency (80%) with 19.9 shoots per callus clump. BAP was significantly better than KN and TDZ for inducing shoot bud formation. Experiments were conducted with KN and TDZ to test their ability of shoot regeneration, the range of 2.0mg/l KN and TDZ singly produced 18.8 and 15.7 shoots per callus clump with high frequency of 77.1 and 71.4%, respectively (Table - 4.3). Increasing concentration of BAP, KN and TDZ up to optimum level increased the rate of shoot proliferation and further increase of concentration of PGR, showed a decline of shoot proliferation. Addition of lower concentration of NAA (0.6mg/l) with optimum concentration of BAP or KN enhanced the proliferation of shoots. Higher concentration of NAA (1.2mg/l) showed reduction in shoot number. MS medium supplemented with BAP (2.5mg/l) and NAA (0.6mg/l) produced the highest frequency (88.6%) of shoots (26.8) regeneration and KN (2.0mg/l) + NAA (0.6mg/l) produced highest frequency (80%) of shoots (23.3) regeneration (Plate - 4.4).

4.4.2.3. Regeneration of plant from Internode derived callus

For shoot induction and proliferation, the light green, less compact or friable callus was transferred onto MS medium supplemented with different concentrations of BAP/KN/TDZ at 1.0 to 3.5mg/l alone, it became greenish nodular and more organized. Adventitious shoot bud was observed from the surface of the callus within 10 – 20 days of inoculation. Formation of leaves and shoot elongation occurred within a month. The highest regeneration frequency (77.1%) and maximum number of (18.2 ± 2.90) shoots was achieved on 2.5mg/l BAP whereas KN and TDZ alone was comparatively with lesser regeneration frequency (74.3 and 68.6%) and maximum number of (16.8 ± 2.24 and 14.8 ± 2.17) shoots was achieved on 2.0mg/l KN and TDZ, respectively. Upon increasing concentration of BAP, KN and TDZ above the optimal level, a gradual decrease in regeneration ability was noticed (Table - 4.4).

In the second set of experiment, regeneration potential of callus tissue was tested on MS medium supplemented with optimal concentration of BAP (2.5mg/l) or KN (2.0mg/l) with different concentrations of NAA (0.2 - 1.2mg/l). Shoot buds generally
arose as clusters within the callus as well as on abaxial surface of callus on MS medium supplemented with BAP (2.5mg/l) and NAA (0.6mg/l) and gave maximum shoot morphogenic frequency (82.9%) with maximum number of (23.8 ± 2.79) shoots followed by KN (2.0mg/l) with NAA (0.6mg/l) that produced maximum frequency (77.1%) with number of (21.0 ± 2.97) shoots per callus clump (Plate - 4.5).

4.4.2.4. Regeneration of plant from Inflorescence rachis and Root derived callus

Inflorescence rachis and root explants derived calli were transferred to MS medium supplemented with different concentrations of BAP, KN and TDZ individually for induced organogenic potential. Among the various cytokinins tested, BAP was found to be more effective than others with respect to differentiation and subsequent multiplication of shoots from callus clump. Of various levels of BAP tested, 2.5mg/l BAP proved to be the most effective, as in this medium an average of 15.3 ± 2.14 shoots were developed from inflorescence rachis derived callus with 71.4% of culture frequency followed by root derived callus which showed an average of 12.3 ± 2.56 shoots with 68.6% of culture frequency. Comparatively lesser shoot regeneration was obtained on MS medium containing KN and TDZ alone than BAP. An average of 14.5 and 11.2 shoots per callus was obtained using KN (2.0mg/l) followed by TDZ (2.0mg/l) which produced maximum of 12.4 and 9.26 shoots per inflorescence rachis and root callus clump, respectively. Upon lower and higher concentrations of each cytokinins from the optimal level, a reduction in the number of shoots per both callus clump were recorded (Table - 4.5 and Table - 4.6).

The efficiency of optimum concentration BAP (2.5mg/l) or KN (2.0mg/l) with different concentrations of NAA (0.2 – 1.2mg/l) also was evaluated for morphogenetic regeneration of calli. The BAP (2.5mg/l) with NAA (0.6mg/l) was found to be most effective combination for shoot bud regeneration. Inflorescence rachis culture showed maximum frequency (80%) with an average number of 18.0 shoots per callus clump followed by root culture which showed maximum frequency (74.3%) with an average of 16.1 shoots per callus clump were recorded. The KN (2.0mg/l) with NAA (0.6mg/l) produced lesser (16.5 and 14.2) shoot regeneration than BAP + NAA which was obtained from inflorescence rachis and root derived callus, respectively. Shoot regeneration was not observed in every segment of the root. Upon increasing concentration of NAA above optimal level, a gradual decrease in regeneration frequency and low number of shoots per inflorescence rachis and root callus clump were recorded (Plate- 4.6 and Plate- 4.7).
4.4.2.5. Regeneration of Plant from Apical bud Explants

Apical bud explants were cultured on MS medium supplemented with different concentrations of 2,4-D and NAA (1.0 – 3.0mg/l) individually or in combination with BAP/KN (0.2 – 1.2mg/l) for callus induction, but shoots did not develop. The explants enlarged and formed greenish callus from cut base end. The callus growth was best in the presence of 2.0mg/l 2,4-D followed by NAA. The nature of callus was light green and friable in 2,4-D and green and less compact in NAA. There were statistically significant differences observed between callus induction, various auxin types per responding culture. The highest frequency of callus induction (91.4 and 85.7%) was observed on MS basal medium containing 2.0mg/l NAA with BAP/KN (0.6mg/l) alone, respectively (Table - 4.7).

The calli were transferred to shoot bud regeneration medium containing different concentrations of either BAP/KN (1.0 – 3.0mg/l) or in combination with NAA (0.2 – 1.2mg/l) for shoot bud differentiation. Addition of cytokinins (BAP/KN) suppressed callus formation and induced shoot bud differentiation. The maximum of callus produced shoot bud primordia developed into normal shoots within 30 - 40 days of culture. Highest frequency of shoot bud regeneration (85.7%) with an average of 35.2 ± 3.23 shoots was observed on MS basal medium containing BAP 2.5mg/l and NAA 0.6mg/l followed by KN (2.0mg/l) and NAA (0.6mg/l) which produced an average of 29.6 ± 3.35 shoots per callus (Table - 4.8). The shoot buds were subcultured in same medium for maximum shoot multiplication. The mean number of shoots increased with increasing concentration of BAP upto 2.5mg/l and KN 2.0mg/l and well developed shoots were isolated from the tubes and subcultured into elongation medium (Plate - 4.8).

4.4.2.6. Regeneration of Plant from Axillary bud Explants

Axillary bud explants were cultured on MS medium augmented with different concentrations of 2,4-D and NAA individually. After 3 weeks of inoculation all the cultured axillary bud segments showed growth of callus from cut end and then covered the segments. The explants expanded twice of their original size and formed green or light green callus. The highest frequency of (80%) callus induction was observed on a medium containing NAA 2.0mg/l followed by 2,4-D and produced 77.1% of callus induction. Addition of BAP/KN at low concentration (0.6mg/l) with NAA or 2,4-D enhanced callus formation.
formation of 88.6, 85.7, 80.0 and 77.1% was obtained using NAA + BAP, NAA + KN, 2,4-D + BAP and 2,4-D + KN, respectively (Table - 4.7). The axillary bud explants produced highly proliferating, light green and compact nodular calli which were subcultured on MS medium containing different concentrations of BAP/KN and formulation of adventitious shoot buds was noticed from surface of the callus within 4 to 5 weeks of culture (Plate - 4.9). The highest shoot regeneration frequency (77.1%) with the highest number of (23.5) shoots per callus clump was obtained at a concentration of 2.5mg/l BAP (Table - 4.9). The addition of NAA with optimal concentration of BAP/KN significantly increased frequency (82.9 and 80%) of 29.8 and 25.6 shoots per callus clump, respectively using BAP (2.5mg/l) + NAA (0.6mg/l) and KN (2.0mg/l) + NAA (0.6mg/l).

4.4.2.7. Regeneration of Plant from Node Explants

Callus induction was observed from the base of the node and on wounded surface of segments of complete node within 2 - 3 weeks on MS medium containing 2,4-D/NAA individually or in combination with BAP/KN. Explants cultured on growth regulators free MS medium became negative or showed no signs of active growth. Optimum concentration of NAA or 2,4-D at 2.0mg/l singly showed 85.7 and 80% frequency of callus induction and optimum callus frequency (88.6 and 82.9%) was observed using NAA 2.0mg/l with BAP/KN (0.6mg/l), respectively (Table - 4.7). Callus tissue transferred to different concentrations of BAP/KN (1.0 – 3.5mg/l) fortified media became nodular and was found highly competent for shoot bud initiation. In all shoots inducing media containing BAP or KN singly, induction of multiple shoot buds as well as regeneration of shoots were readily achieved. Among the various concentrations of BAP and KN tested, the highest shoot regeneration frequency (77.1%) and highest number of 23.9 ± 3.32 shoots were recorded at 2.5mg/l of BAP. Increasing concentration of cytokinin from optimum level resulted in a decrease in the rate of shoot regeneration ability.

In the second set of experiment, regeneration potential of callus tissue was tested on MS fortified with optimum level of BAP (2.5mg/l) or KN (2.0mg/l) with different concentrations of NAA (0.2 – 1.2mg/l). Shoot bud gradually arose as clusters within the callus as well as on abaxial surface of the callus on MS medium fortified with BAP 2.5mg/l + NAA 0.6mg/l and this combination gave maximum shoot morphogenic frequency (85.7%) with number of 30.1 ± 3.58 shoots followed by 2.0mg/l KN + 0.6mg/l.
NAA which produced moderate frequency of shoot morphogenesis (Table - 4.10). Clearly, NAA showed the synergistic effect with BAP and enhanced the induction of shoot buds from callus and increased the shoot morphogenic response as compared to the combination of other cytokinin (KN) with NAA (Plate - 4.10).

4.4.2.8. Regeneration of Plant from inflorescence bud Explants

Callus formation was demonstrated on inflorescence bud explants taken from field grown plants. The inflorescence bud cultured on MS medium supplemented with NAA/2,4-D (2.0mg/l) alone or in combination with BAP/KN (0.6mg/l) gave rise to luxuriantly growing callus at the basal cut end. Condition and medium provided were optimized for encouraging callus proliferation (Table - 4.7). Friable organogenic callus developed directly from the inflorescence bud cuttings were periodically subcultured on shoot proliferating medium. However, the greenish yellow or light green callus turned green only on regeneration medium, just before shoot regeneration.

The regeneration of callus occurred on MS medium containing different concentrations of BAP/KN (1.0 – 3.5mg/l) individually or in combination with NAA (0.2 – 1.2mg/l). All the media that were used induced callus regeneration and number of shoots per culture varied with type of plant growth regulators and concentration employed. Maximum response was obtained on MS medium supplemented with 2.5mg/l BAP and 0.6mg/l NAA. This combination produced maximum frequency (80%) and number of (25.7 ± 3.46) shoots per callus clump whereas KN (2.0mg/l) + NAA (0.6mg/l) showed maximum of 74.3% responsive culture with 23.4 shoots per callus clump. Comparatively low frequency with less number of shoot regeneration was obtained using BAP and KN alone than combination with NAA (Table - 4.11 and Plate - 4.11).

4.4.3. Shoot Elongation

Shoot elongation varied with hormonal treatments. A significant variation was observed among the elongated shoots of different treatment media. The regenerated shoots were normal when cultured in regeneration medium but when transferred to shoot elongation medium containing either GA₃ alone or in combination with BAP/KN/NAA, the shoot length increased showing healthy and vigorous shoots. The percentage of response, shoot length, number of node and leaves were the greatest on half strength MS medium supplemented with 0.6mg/l GA₃, only when used individually. Here a maximum
of 82.9% culture response with an average of 7.16 cm mean shoot length, 7.47 nodes and 14.3 leaves were observed. The addition of KN along with GA₃ (0.6mg/l) significantly increased the percentage of response as well as average of shoot length, number of nodes and leaves per shoot. The optimum response was observed on MS medium fortified with 0.5mg/l KN and 0.6mg/l GA₃. On this medium, 91.4% of culture responded with an average of 8.37 cm shoot length, 7.95 nodes and 15.6 leaves per shoot were recorded (Table - 4.12 and Plate - 4.3 to 4.11). Comparatively, GA₃ + BAP and GA₃ + NAA combinations showed low frequency response with less shoot length, nodes and leaves. The balance between auxin and cytokinin very much influenced morphogenic responses of elongated shoots.

4.4.4. Rooting, Hardening and Acclimatization of in vitro raised shoots

Individual shoots at least 2 – 3 cm long were separated and cultured on half strength MS medium supplemented with IBA or IAA (0.1 – 2.5mg/l) for root induction. The shoots that were below 1.0 cm in length did not produce any roots when cultured on rooting medium. The shoot produced roots at all concentrations of IBA and IAA (Table - 4.13) but the highest efficiency of 88.6% rooting was observed in 1.5mg/l IBA. The development of an average number of 19.7 roots with 9.3 cm root length from a single shoot was noticed in 1.5mg/l IBA fortified medium at the end of the 20 – 25 days of culture. The IBA was comparatively better than IAA, IBA + KN and IBA + BAP in terms of both percentage of the cultures that responded as well as number of roots and root length per shoot (Plate - 4.3 to 4.11).

The rooted shoots were successfully transplanted to paper cups containing garden soil, river sand and saw dust in the ratio of 1:1:1 and the humidity was maintained at approximately 80 – 90% by covering with polythene bag. The plants were watered twice every four days. After two months plants were transferred to large pots and after acclimatization, the 100 days old plants were transferred to the field.

4.5. DISCUSSION

Recent advances in plant tissue culture technology have opened up many new avenues for conducting basic genetic research on higher plants at the cellular level and have provided potentially powerful new tools in the hands of plant breeders for generating, selecting and propagation of novel and economically important plant varieties. The use of
in vitro technology in the mass propagation of aromatic and medicinal plants has been gaining momentum in the recent years (Parida et al., 2005). A plant regeneration system via., indirect shoot organogenesis has been established in this study and to describe a new procedure for shoot regeneration in Plectranthus barbatus. Explants were taken from in vivo and in vitro shoot cultures. Conditions encouraging callus proliferation and recovery of large number of adventitious shoots were identified.

Application of in vitro techniques have been routinely practiced for the multiplication of many medicinal plants to meet the demand of pharmaceutical firms and to protect the natural populations of rare and endangered plant species (Cuenca and Marco, 2000; Reddy et al., 2001; John Britto et al., 2002; Sharma and Wakhlu, 2003; Govindaraju et al., 2003; Nagaraja et al., 2003; Prakash et al., 2004; Molina, 2004; Aoshima, 2005; Filipecki et al., 2005; Faisal et al., 2006; Moghaieb et al., 2006; Feeney et al., 2007; Shen et al., 2007; Jones et al., 2007; Rathod et al., 2008; Meratan et al., 2009 and Shilpa et al., 2010).

4.5.1. Callus Induction

The essential findings of Skoog and Miller (1957) that organogenesis in tissue cultures is governed by the balance of auxin and cytokinin in the medium cannot be demonstrated universally due to the explant sensitivity or the original content of endogenous growth regulators. Methods have been developed for reproducible plant regeneration system from cultured explants of P. barbatus. Results obtained from this experiment revealed that the explants vary in their different hormone requirements for callus induction, shoot bud regeneration, multiple shoot formation and root initiation.

The explants were cultured on MS basal medium fortified with different concentrations of auxins (2,4-D, NAA and IAA) or in combination with cytokinins (BAP, KN, TDZ AdS and CW). In the present study, three auxins with five cytokinins combinations were tested for their callusing ability. NAA (2.0mg/l) and BAP (0.6mg/l) combination was in general observed to be more effective than other combinations for callus initiation within 15 days of culture. Our results indicated that the addition of cytokinin effected with an increase in the frequency of callus induction.
The callus induction frequency was different with different auxins. So, the present study clearly indicates that the auxin type greatly influenced the callus induction frequency. Of the three auxins individually tested, NAA induced optimum callus followed by IAA and 2,4-D within two to three weeks. In the present study, the callusing frequency increased with an increase in the concentration of auxin upto 2.0mg/l supplemented with cytokinin (BAP/KN) 0.6mg/l in the medium. The callus induction frequency increased in all the (nine) explants with three auxin combinations tested in the present study. This is consistent with reports on the tissue culture study of different explants (Puchooa 2004; Shen et al., 2007; Rathod et al., 2008; Debnath, 2008a and Arellano et al., 2009).

Similar results were also obtained on MS medium supplemented with 2,4-D, NAA and IAA alone or in combination for callus induction in the past by Chen et al. (2005), Gopi and Vatsala (2006) and Meratan et al. (2009). The auxin alone or in combination with cytokinin supporting callus growth has been reported by Murnilawati et al. (2006), Anand et al. (2001) and Saradamani et al. (2003). Moreover the combination of auxin in high concentration and cytokinin in low concentration was more effective for callus formation. Similar results were obtained in Salvia canariensis (Molina, 2004), Ocimum basilicum (Sudhakaran and Sivasankari, 2003), Heracleum candicans (Sharma and Wakhlu, 2003) and Nicotiana tabacum (Murnilawati et al., 2006). In our experiment it is seen that NAA 2.0mg/l and BAP 0.6mg/l had optimum frequency of callus formation which was observed within 2 to 3 weeks of culture.

The proliferation efficiency of callus of nodal explant was significantly higher than that of leaf explant for four to five incubation of culture (Gopi and Vatsala, 2006). In the present study, callus induction response varied with the type of explants and hormone. Among the nine explants investigated, node and internode explants showed maximum response followed by petiole, inflorescence rachis, apical bud, axillary bud, inflorescence bud, leaf and root. The differential response of callus induction from the explant was also previously reported by Lisowska and Wysokinska (2000), Feeney et al. (2007), Muthuramu et al. (2007) and Sriskandarajah et al. (2008). Morphology, texture and colour of the callus were depending on the nature of hormones used. Auxin (2,4-D, NAA and IAA) individually resulted in greenish white or greenish yellow and less compact or friable calli whereas in combination with cytokinin (BAP/KN/ADS/TDZ and or CW)
embryonic light green calli were light brown and whitish brown in colour with nodular, less compact calluses. These results corroborate with the previous findings of Chen et al. (2005) in Narcissus tazetta, Withania somnifera (Govindarasu et al., 2003) and Gymnema sylvestre (Gopi and Vatsala, 2006). Callus growth of P. barbatus was highly influenced by concentrations of growth regulators and coconut water (CW) added to the medium. Baskaran and Jayabalani (2005) and Baskaran et al. (2006) also reported that the addition of CW (10%) with 2,4-D proved to be better for callus growth. Similar results were observed in the present study and the low concentration of 2,4-D (1.5mg/l) or NAA (2.0mg/l) with CW (15%) was effective for callus induction.

4.5.2. Shoot bud Differentiation and Multiple shoot Regeneration

In the present study, we have obtained increased shoot bud regeneration efficiency from different explants derived callus by culturing them on MS basal medium containing different concentrations of BAP/KN/TDZ (1.0 – 3.5mg/l) with low levels (0.2 – 1.2mg/l) of NAA. In the absence of cytokinin, however, there was no induction of shoot bud regeneration. Muthuramu et al. (2007) reported that shoot regeneration was indirect and developed through calli. Auxins and cytokinins are the two types of phytohormones most often needed in culture and a relatively high ratio of cytokinin (2.0mg/l), auxin (0.5mg/l) favored shoot formation. This is in agreement with the present study whereas BAP (2.5mg/l) with NAA (0.6mg/l) were as effective for shoot formation. Similar results were also reported by Murashige and Skoog (1962) who observed that a relatively low auxin; cytokinin ratio induced shoot formation in tobacco callus.

In this study, 85.7% of apical bud callus explants formed shoots on MS medium fortified with BAP (2.5mg/l) + NAA (0.6mg/l) within 4 weeks of culture. In contrast, Oh et al. (1991) achieved a much lower frequency of shoot formation on callus explants after 4 weeks on half strength MS medium supplemented with either 1.0mg/l BAP (25%) or 0.1mg/l BAP (30%). Frequency of shoot formation in leaf explant varied between 22.9 – 88.6 %, and the lowest frequency was observed on medium containing 3.5mg/l TDZ and higher frequency with 2.5mg/l BAP + 0.6mg/l NAA. This is in contrast with that of Meratan et al. (2009) who achieved an optimum frequency (78.6%) of shoot formation on medium containing NAA + TDZ and lowest frequency (6.56%) of shoot formation on 2,4-D + KN fortified medium. Abubacker and Murugesan (1999) have reported the
concentration of BAP (1.5mg/l) and NAA (0.5mg/l) to be most congenial for shoot regeneration in young stem explants followed by Nirwan and Kothari (2004) who have reported that BAP (2.0mg/l) and IAA (0.5mg/l) to be effective for shoot bud regeneration in apical meristem explants of *Sorghum bicolor*. But in the present investigation BAP 2.5mg/l + NAA 0.6mg/l was effective for regeneration in all the explants derived callus especially apical bud derived callus which produced optimum of 35.2 shoots per callus clump in this combination.

Higher concentration BAP (above 3.0mg/l) reduced the percentage of response, number of shoots and shoot length. This is an agreement with previous findings of Baskaran *et al.* (2006) who observed the same results in *Sorghum bicolor*. Significant effects of subsequent shoot regeneration from calli on MS medium fortified with BAP 2.5mg/l alone was more effective than KN and TDZ because less shoot regeneration was observed on MS based medium supplemented with 2.0mg/l KN and 2.0mg/l TDZ alone in all the explants derived callus. In contrast, Shen *et al.* (2007) have shown that 2iP was more effective than KN. Voyiatzi and Voyiatzis (1989) also found that 2iP was more effective in inducing lateral shoot multiplication in *Dieffenbachia* than KN.

No morphogenetic response was observed from callus clumps cultured on MS basal medium which was devoid of plant growth regulators. Callus tissue transferred to different shoot bud induction media became nodular and were found to be highly competent for shoot bud initiation. Similar results were obtained by Faisal *et al.* (2006) in *Ruta graveolens*, Parida *et al.* (2005) in *Pogostemon cablin* and Jones *et al.* (2007) in *Echinacea purpurea* in all the shoots inducing media containing BAP or KN or TDZ singly, and induction of multiple shoot buds as well as regeneration of shoots were readily achieved. Increasing the concentration of cytokinin from 1.0mg/l to 2.0mg/l or 2.5mg/l resulted in an increase in the rate of shoot regeneration ability and further increased concentration gradually decreased the shoot numbers. Similar results were earlier reported on *Coleus forskohlii* (Reddy *et al.*, 2001) and *Tylophora indica* (Faisal and Anis, 2005) where the higher concentration decreased shoot multiplication. It is in contrast to reports of Faisal *et al.* (2006) where an increase in the concentration of BAP from 1.5 – 2.5mg/l resulted in a decrease in the rate of shoot regeneration. Addition of NAA showed the
synergistic effect of with BAP and enhanced the induction of shoot bud from callus and increased the shoot morphogenetic response as compared to the other combinations of NAA + KN. A similar observation is also reported in Petasites hybridus (Wldi et al., 1998), Eucalyptus grandis (Luis et al., 1999), Coleus forskohlii (Reddy et al., 2001), Tylophora indica (Faisal and Anis, 2003) and Sarcostemma brevistigma (Thomas and Shankar, 2009).

The superiority of BAP over other cytokinins like KN and TDZ for shoot proliferation was also established in other plants such as Morus nigra (Yadav et al., 1990), Plumbago zeylanica (Sahoo and Debata, 1998), Lathyrus sativus (Zambre et al., 2002), Anisomeles indica (John Britto et al., 2002), Sorghum bicolor (Saradamani et al., 2003), Litchi chinensis (Puchooa, 2004), Centella asiatica (George et al., 2005) and Phaseolus vulgaris (Arellano et al., 2009). Variations among the wheat varieties for regeneration of plants from callus has been reported by Viertel et al. (1998) and Talukder et al. (2004). In present study, varietal differences for regeneration ability of the plants from different calli were observed. Among the different calli apical bud, node and axillary bud derived calli produced maximum number of shoots followed by leaf and petiolar calli which produced moderate shoot regeneration. There is a great deal of variability between plants and the growth regulators necessary for morphogenesis and the growth regulator requirement of each type of explants which should be determined (Hussey, 1975; Hughes, 1981 and Puchooa, 2004).

Chen et al. (2005) have reported the regeneration of Narcissus tazetta from anther callus on MS medium supplemented with 0.5mg/l 2,4-D and 1.0mg/l BAP. High frequency of shoot regeneration from callus of Cichorium intybus on MS medium fortified with BAP (1.0mg/l) and IAA (0.2mg/l) was reported by Velayutham et al., (2007). Arellano et al. (2009) observed that the optimum response for shoot regeneration from calli was on MS medium supplemented with BAP 5.0mg/l. In the present investigation for regeneration, when a cytokinin was used singly in the culture medium, there was low frequency of differentiation. Maximum regeneration capacity was observed when cytokinin with auxin were used after three to four weeks of culture. This result corroborates with the previous finding of Puchooa (2004).
However in the present study, the yellowish callus turned green on regeneration medium just before shoot regeneration. The regeneration of callus occurred on MS medium supplemented with different concentrations of KN and TDZ (1.0 – 3.5mg/l) alone or in combination with NAA (0.2 – 1.2mg/l). All the media used induced callus regeneration, but the percentage of regeneration and number of shoots per culture varied with the type of explants, growth regulators and concentrations employed. Maximum response was obtained on MS medium supplemented with 2.5mg/l BAP and 0.6mg/l NAA in all the explants followed by KN (2.0mg/l) and NAA (0.6mg/l). The synergistic action of auxin and cytokinin for shoot regeneration from callus is well documented in several published reports Saxena et al. (2000), Zambre et al. (2002), Wei et al. (2006) and Thomas and Shankar (2009).

4.5.3. Shoot Elongation

Maximum shoot elongation was obtained on MS medium without cytokinin. In fact shoot elongation decreased with increasing concentration of cytokinin as has been reported (Cuenca et al., 1999). Yadav and Padmaja (2003) reported that the shoots when transferred to MS medium fortified with 1.0mg/l BAP for 4 weeks favoured shoot elongation. Shoots regenerated on BAP supplemented medium showed slow growth and poor internodal elongation in comparison to the shoot regeneration from KN supplemented medium which revealed better shoot elongation (Faisal and Anis, 2005). Anand et al. (2001) achieved higher rate of shoot elongation on MS medium containing BAP with GA₃. The present study reveals some distinct advantages over previous reports. In our study, shoot elongation occurs when the shoot bud (above 2.0 cm) was transferred to half strength MS medium supplemented with GA₃ alone or in combination with KN or BAP or NAA. All the media used induced shoot elongation. Optimum frequency (91.4%) of shoot elongation (8.37 cm shoot length) was obtained on half strength MS medium fortified with GA₃ (0.6mg/l) + KN (0.5mg/l) followed by GA₃ (0.6mg/l) alone which produced moderate frequency (82.9 %) with 7.16 cm shoot length. This is consistent with the reports of Grigoriadou et al. (2002) and Govindaraju et al. (2003).

4.5.4. Rooting, Hardening and Acclimatization of in vitro Shoots

The in vitro regenerated shoots were isolated and cultured for root induction on full and half strength MS basal medium. Among these, half strength MS medium was found sufficient for the induction of roots. The successful application of half strength MS
medium for root inducing experiment have also been documented in various *in vitro* protocols *viz.*, *Coleus forskohlii* (Reddy et al., 2001), *Sesbania drumondii* (Cheepala et al., 2004), *Tylophora Indica* (Faisal and Anis, 2005), *Ruta graveolens* (Faisal et al., 2006) and *Sarcostemma brevistigma* (Thomas and Shankar, 2009). Half strength MS medium supplemented with different auxins were also used to enhance root formation. Of the two auxins (IBA and IAA) used, IBA 1.5mg/l was more effective for root formation than IAA. These media produced an average of 19.7 roots with 9.3 cm root length. This result is in agreement with the findings of Agarwal and Sardar (2006) on *Cassia angustifolia*, Sharma *et al.* (2004b) on *Hordeum vulgare*, Anand *et al.* (2001) on *Vigna unguiculata* and George *et al.* (2005) on *Centella asiatica*. In the present study, IAA (1.5mg/l) showed maximum of 12.3 roots per shoot with average of 6.3 cm root length. This is in accordance with previous reports of Saradamani *et al.* (2003) and Baskaran *et al.* (2006).

The regenerated plantlets with well developed shoots and roots were transferred to pots contains sterilized garden soil, river sand and saw dust for hardening at different light (16/8 hrs photoperiod) conditions (Faisal and Anis, 2003). The plants were covered with polythene bags to maintain humidity and watered with autoclaved tap water (Reddy *et al*., 2001). After a month they were transferred to greenhouse and all the plants exhibit normal morphology with respect to growth habit, leaf shape, flower shape and size when compared with the mother plant.

The present communication presents an efficient method for large-scale mass propagation of *P. barbatus* through a dedifferentiated callus state. This *in vitro* procedure should be suitable for conservation and large-scale propagation of aromatic and medicinal plants of *P. barbatus*. 