Chapter VI

6. SUMMARY AND CONCLUSION

6.1 Micropropagation of bamboo (*Bambusa arundinacea* (Retz.) Wild. and *Bambusa nutans* Wall. Ex.Munro) using nodal explants

The present study reveals a reliable protocol for *in vitro* propagation that could be applied for commercial production of two edible bamboo species. A faster and easier methodology for the commercial production of these bamboo species by *in vitro* has been developed with standardization of protocol for rapid multiplication will help to face the considerable demand of paper and pulp industry and this will also enable these species with medicinal properties for higher productivity.

High frequency of multiple shoot bud induction and plant regeneration was achieved using *in vitro* seedlings derived from nodal explants of *B. arundinacea*. From both the experiments I and II, experiment II was noticed with maximum plant regeneration using various factors for high frequency plant regeneration. The combination of BAP (3.0 mg/L) and IBA (0.5 mg/L) was found to be best for highest percent of shoot bud initiation (87.2 %), with more number of shoots (24.2 shoots/explant). The highest frequency (95.2%) of shoot bud multiplication with maximum number of shoots (90.5 shoots/culture) was noticed on a medium containing 4% CW with 4% sucrose. Maximum shoot length of about 5.8 cm was noticed on MS medium augmented with 3.0 mg/L BAP in the presence of ADS (5.0 mg/L). Maximum percent of rooting (85%) was noticed on MS medium augmented with 3.0 mg/L IBA and 2.0 mg/L AgNO₃ after 14 days of culture. Well rooted and healthy plantlets obtained were transferred to the green house for further field establishment. The plant survival rate noticed was 92%.

Also an efficient method for *in vitro* plant regeneration in *B. nutans* using nodal explants from field-grown culms was developed on MS basal medium augmented with different concentrations of BAP (0.5-2.5 mg/L) and KIN (0.5-2.5 mg/L) alone for shoot bud induction and multiplication. The highest frequency of shoot regeneration (83.5%) was noticed on MS medium containing 2.0 mg/L BAP with11.62 shoots/culture. Initial exposure of shoot to IBA (4.0 mg/L) with glucose
(1.5%) and subsequent withdrawal of auxin from the medium was found to be very effective for root development. Highest frequency of rooting (78.3%) was noticed on MS medium augmented with 4.0 mg/L IBA. Healthy plantlets obtained were transferred to the green house for further field establishment with 85% survival rate.

Among, the two bamboo species, B. arundinacea was found to be the best and efficient for large scale plant production derived from the nodal explants. The protocol described herein has been well developed and the hardened plantlets have been established in the field condition - Biodiversity garden in Periyar University. Both the micropropagation protocol can be utilized for large scale production of these superior genotypes as well as conservation of these medicinally important species Bambusa arundinacea and Bambusa nutans in the near future.

6.2 Somatic embryogenesis and plant regeneration from mature seed embryo explants of Bambusa arundinacea (Retz.) Wild.

Efficient plant regeneration via somatic embryogenesis was achieved using mature seed embryo explants of Bambusa arundinacea. The mature seed embryo explants were cultured on MS medium supplemented with different concentrations of 2,4- dichlorophenoxy acetic acid (2,4-D) (0.5-4.0 mg/L) in combination with 1.0 mg/L BAP for induction of embryogenic callus. Maximum percent of embryogenic callus induction (85%) was obtained on MS medium containing 1.0 mg/L 2,4-D and 1.0 mg/L BAP combination. The embryogenic callus noticed was compact with nodular structures. The nodular embryogenic calli were subcultured onto MS medium supplemented with various concentrations of BAP (0.5-4.0 mg/L) and/or KIN (0.5-4.0 mg/L) in combination with 1.0 mg/L each of 2,4-D and α - naphthalene acetic acid (NAA) for maturation of somatic embryos and plant regeneration. The combination of 2,4-D (1.0 mg/L) + NAA (1.0 mg/L) + BAP (1.0 mg/L) was found to be best for somatic embryo maturation (94%). Matured somatic embryos were cultured on MS medium containing different concentrations of BAP/KIN (0.5-4.0 mg/L) in combination with 1.0 mg/L NAA for embryo germination and plantlet development. Of the two cytokinins tested, BAP at 1.0 mg/L was found to be best for maximum percent of embryo germination (88.3%) than KIN. The germinated plantlets were successfully established in the plastic cups for acclimatization. After
two weeks, plantlets were transferred to the greenhouse condition and subsequently established in the field where 90% plantlets were survived. In addition, the morphology of the somatic embryos was confirmed by histology and SEM analysis. In this study, a reliable protocol for plant regeneration via somatic embryogenesis was achieved using mature seed embryo explants and it could be used for commercial scale production of *Bambusa arundinacea* seedlings in the near future.

6.3 Biochemical and Molecular analysis using *in vitro* leaf samples from *Bambusa arundinacea* (Retz.) Wild. and *Bambusa nutans* ex. Munro

The present study clearly revealed that, higher yield of isovitexin was obtained by methanol extraction method followed by quantitative HPLC analysis from the leaves of *B. arundinacea* and *B. nutans*. While comparing the quantitative data's obtained, *in vitro* raised leaf samples of *B. arundinacea* showed the highest level of isovitexin content (2.53 mg/g DW) with that of *B. nutans* (2.06 mg/g DW). The results indicate that isovitexin has the potential to be used as an anticancer drug in near future. The uniform banding profile among the tissue culture raised progenies, confirm genetic stability in plant raised through micropropagation in both the *Bambusa arundinacea* and *Bambusa nutans* species. The results confirmed that the regenerated plants were true to type in nature. The genetic diversity between the bamboo species from various locations in Tamil Nadu was studied and polymorphism was also recorded using RAPD technique and the phylogenetic tree was constructed. The results of this present study on genetic diversity revealed that the genetic variation and evolutionary dynamics of both the bamboo species. Overall, these markers were informative in differentiating the various bamboo accessions and determining the level of genetic variation within and among the genera.

In summary, the results from this study indicate that the RAPD technique is a useful tool for the identification of germplasm analysis and genetic relationships between and within the bamboo species. The relatively large number of polymorphisms obtained seems due to large phylogenetic distance among these taxa. It would allow a more quantitative assessment of genetic distances between
Bambusa species. Present analysis, together with data from other classical methods, could thus be used to make a more accurate reconstruction of the bamboo evolution. Furthermore, this approach might be helpful in identifying taxa of potential value in genetic improvement programmes.

6.4 Phytosynthesis of silver nanoparticles using in vitro and in vivo leaf extracts of Bambusa arundinacea (Retz.) Wild. and Bambusa nutans Wall. ex. Munro and to evaluate its antibacterial as well as cytotoxicity effects against PC3 cancer cell lines.

Bamboo species possess various bioactive compounds which showed anticancer activities. The present study reports the biosynthesis of metallic silver nanoparticles (AgNPs) from silver ions using in vitro and in vivo grown leaf samples of Bambusa arundinacea and Bambusa nutans and its antibacterial activity as well as anticancer activity against human prostatic cancer cell lines (PC3). The metallic silver nanoparticles were synthesized at room temperature by treating the leaf extracts with 2 mM silver nitrate by using boiled method. Water-soluble organics present in the plant materials were mainly responsible for the reduction of silver ions to nano sized Ag particles. The UV–Visible spectrum showed an absorption peak at 365 nm, which reflects surface plasmon resonance (SPR) of AgNPs. Results clearly showed that the bamboo leaf extracts containing prominent FTIR peaks of biomolecules and functional groups that act as capping agents and involved in the stabilization of the synthesized nanoparticles. The face-centered cubic (fcc) crystalline nature of the synthesized Ag nanoparticles was confirmed by the strong diffraction peaks observed under X–ray diffraction (XRD). X-ray diffraction analysis revealed the distinctive facets (210, 122, 231, 142, 220 and 311 planes) for silver nanoparticles. The surface morphology of the AgNPs was determined by SEM and the shape of the nanoparticles was found to be spherical. EDX analysis of the nanoparticles dispersion was confirmed the presence of silver metal ions in the range of 2–4 keV. Transmission electron microscopy (TEM) photography showed biosynthesized AgNPs were predominantly spherical in shape with an average size of 30-90 nm. The nanoparticle synthesized using in vitro leaf extracts of B. arundinacea by boiling method showed effective antibacterial activity than the B. nutans leaf extracts against E. coli, Bacillus subtilis, Staphylococcus aureus.
followed by *Pseudomonas aeruginosa*. Cell viability assays were carried out to determine the cytotoxicity effect of AgNPs synthesized from *in vitro* leaf extracts of *Bambusa arundinacea* and *Bambusa nutans* on PC3 human prostate cancer cell line and VERO normal African monkey kidney cell line. The inhibitory concentration (IC$_{50}$) values of silver nanoparticles for *Bambusa arundinacea* and *Bambusa nutans* were found to be 73.57 µg/mL and 84.88 µg/mL and Vero cells were found to be 93.58 µg/mL and 96.41 µg/mL respectively. An induction of apoptosis was proved by (AO/EtBr) staining. The percentage of the apoptotic bodies was found to be 76 % and 62 % for *in vitro* leaf extracts of *B. arundinacea* and *B. nutans* synthesized silver nanoparticles respectively. The results strongly revealed that the synthesized silver nanoparticles from *B. arundinacea* had potential anticancer activity against human PC3 cell lines while compared with *B. nutans*. These results confirmed the presence of nano-crystalline silver particles. *B. arundinacea in vitro* raised leaf samples proved to be superior while comparing with that of *B. nutans* for antibacterial as well as cytotoxicity activity. The present study supports the potential of isovitexin from *Bambusa arundinacea* leaf extract as an anticancer agent for PC3 cancer cell lines and can be used commercially in future.

6.5 Summary

In summary, *in vitro* propagation of two edible bamboo species *Bambusa arundinacea* and *Bambusa nutans* were developed using nodal explants. *In vitro* propagation of two bamboo species was achieved by standardizing a reliable protocol, and establishment of culture medium with contaminant free cultures. Optimization of continuous multiple shoot was achieved and the hardened *in vitro* plantlets were well established in Biodiversity garden Periyar University. Large scale *in vitro* plant regeneration through somatic embryogenesis was achieved from mature seed embryos of *Bambusa arundinacea*. An efficient protocol for callus induction, shoot regeneration, rooting was achieved and the acclimatized plantlets were transferred to the field condition. Histological analysis was carried out to confirm the embryogenic nature, further the structural appearance of the embryo was determined in detail by SEM analysis. Genetic stability of the *in vitro* regenerated plantlets was confirmed by DNA fingerprinting studies. The genetic diversity
between the two bamboo species from various locations in Tamil Nadu was studied and polymorphism was recorded using RAPD technique and the phylogenetic tree was constructed. The flavonoid content Isovitexin was quantified using HPLC analysis from \textit{in vitro} and \textit{in vivo} leaf samples of \textit{Bambusa arundinacea}. Phytosynthesis of boiled leaf samples showed best results comparing the centrifuged green synthesis methodology in both the bamboo species. Green synthesis of silver nanoparticles was achieved and the comparative study was done from \textit{in vitro} and \textit{in vivo} leaf samples to evaluate its antibacterial as well as cytotoxicity activity using human PC3 cancer cell lines.

Based on the importance of \textit{Bambusa arundinacea} and \textit{Bambusa nutans} high demand of quality plating material and lack of efficient methods for large scale production, studies were conducted for the development of protocols from nodal explant derived from \textit{in vitro} grown seedlings for further shoot bud proliferation and somatic embryogenesis. Comparative studies on biochemical analysis and evaluation of genetic fidelity of \textit{in vitro} propagated plants through axillary shoot bud proliferation and genetic diversity with polymorphism was carried out and comparative studies were conducted on green synthesis using silver nanoparticles from \textit{Bambusa arundinacea} and \textit{Bambusa nutans}. In view of the nature of the plant, more research can be done to investigate the unexplored and unexploited potential of these pharmaceutically important bamboo species.

In conclusion, an efficient plant regeneration protocol was established. Comparative studies on biochemical analysis and evaluation of genetic fidelity and genetic diversity were carried out. The bioactive compound isovitexin present in \textit{B. arundinacea} leaf extract can be regarded more specific, with high potential to suppress the growth of PC3 cancer cell lines. These results strongly suggest that the synthesized nanoparticles from bamboo leaves can act as anticancer agent and could be used as an alternative anticancer drug commercially in the near future.