SUMMARY
**SUMMARY**

*Pongamia pinnata* (L) Pierre (*Syn*: *Pongamia glabra* Vent), an oil producing tree legume, popularly known as Karanja, is the most widely available non-edible oil tree in India. This multipurpose tree species is well adapted for various agroclimatic conditions and is one of the most extensively chemically investigated plants. Potential of the seed derived oil of *Pongamia* as a substitute for diesel is recognized and often identified as "Bio-diesel" (Vivek & Gupta AK, 2004). A large number of bioactive compounds have been isolated and characterized from various parts of this tree (flavonoids, furanoflavonoids), which has many industrial and medicinal uses (Wealth of India, V.3, 1998; Parmar, 1976).

*Pongamia* can serve as a source of high quality fuel and raw material for industries. Raising plantations of clonally propagated, high seed yielding trees will contribute to increased seed production. These plantations will also serve the purpose of seed orchards for production of superior quality seeds. *Pongamia* tree can be exploited as an alternate source of edible oil by silencing the related genes, which cause production of undesirable substances. For genetic modification, the first pre-requisite is a reproducible *in vitro* regeneration system. There is no literature on *in vitro* regeneration or tissue culture studies in this species.

*Pongamia* species, which represent a substantial variability in phenotype as well as chemo type properties for the oil and its components, do need to be documented well for establishing phylogenetic relationships and unique marker profiles at DNA level. To date, there is no literature available regarding the molecular scaffold of this tree.

The present study was designed with the following objectives:

1. Optimization of conditions for propagation using juvenile tissues.
2. Optimization of conditions for clonal propagation using mature nodal buds.
3. Optimization of protocol for *de novo* morphogenesis (organogenesis and somatic embryogenesis).
4. Assessment of variability/similarity among the trees and fidelity of the *in vitro* raised clones using ISSR markers (Molecular characterization).
The major constraint encountered in the study to establish a regeneration protocol for clonal propagation from seedling explants was to obtain seedlings under aseptic condition. Several factors were tested to obtain microbe free seedlings. Removal of seed coat prior to germination, controlled fungal growth partially but enhanced bacterial growth. Antibiotic Cefotaxime was ineffective in controlling bacterial contamination. Increased germination frequency of dry mature seeds was obtained in media with 8.88µM BA in MS medium. Cotton plugged culture vessels favored germination.

A protocol for in vitro propagation of this plant was standardized using seedling explants. This is the first report on in vitro regeneration of *Pongamia pinnata* (Sujatha and Hazra, *Journal of Plant Biotechnology* 33(4): 263-270, 2006). Seedling derived nodal explants and cotyledon nodes with attached cotyledons were used as explants. Optimum shoot proliferation from nodal explants and cotyledon nodes was achieved in MS medium supplemented with 8.88µM BA and 3% sucrose. Reculturing of cotyledon node explants after removal of shoots produced more shoots from same site. This process was repeated for 8 cycles and 4-8 shoots were obtained in each cycle. This process offers an alternative to produce clones and to avoid the step of repeated seed germination in vitro. The shoots elongated and rooted (75%) in half strength MS basal medium supplemented with 0.22% activated charcoal. Plantlets survived on transfer to soil. The in vitro raised shoots rooted extra vitrum (67.5%) and hardened successfully.

To eliminate the seed borne contamination, cotyledon node explants were isolated from semi mature seeds obtained by dissecting the surface sterilized green pods. These were cultured in varying concentrations of TDZ for induction of shoots from cotyledon node meristem. After 20 days pretreatment in TDZ, cotyledon node explants with intact cotyledon and embryo axis explants responded in culture. On transfer to MS media for 2-4 passages, the buds induced in the cotyledonary meristem differentiated. Optimum TDZ concentration was 11.4 µM. Buds induced in high TDZ (13.6 & 22.7µM) took longer time to differentiate, delaying the rooting step. The shoots were rooted (80%) in half strength MS media supplemented with charcoal. The rooted shoots were hardened (75%) and transferred to green house successfully.

A protocol for clonal propagation of *Pongamia* using mature nodal buds was optimized (Sujatha and Hazra, *In Vitro Cellular and Developmental Biology, 2007 – In press*). Four basal media formulations including MS, SH, B5 and WPM were tested. MS basal media was superior for mature
bud culture of *Pongamia*. Effects of various growth regulators including BA, KN, Z and TDZ were studied on sprouting of nodal buds. No multiples were produced in MS supplemented with BA and KN singly. Supplementing Adenine sulphate (AdS) with BA promoted production of 2-3 multiples from the nodal bud. Media with kinetin and AdS was ineffective. In TDZ, sprouting was completely suppressed and the meristems were swollen. Caulogenic buds appeared from the swelling on withdrawal of TDZ. Number of buds was more in the explants pretreated in increased concentration of TDZ. Clusters of buds elongated on transfer to GR free MS medium. In TDZ 0.45 μM the response was optimum as the buds proliferated in this medium and the induced bud differentiated faster. Shoot cultures were maintained by subculturing in 0.45 μM TDZ. Among the parameters tested for optimization, sucrose at 4% and pH 5.8 were optimum for shoot proliferation and growth. Reculturing of primary explants after cropping the shoots produced more shoots. This process was followed for 6 cycles to obtain additional shoots in each cycle. Shoots elongated and rooted (70%) in GR free MS medium. Rooted shoots survived in greenhouse (65%). Repeated proliferation of caulogenic buds from same origin may find application in the rescue of endangered germplasm and in development of transgenics. This study not only describes a simple protocol for clonal propagation of an important tree species “*Pongamia pinnata*” for the first time, but also a system to study some of the processes of TDZ induced morphogenetic activities.

Deembryonated cotyledon from green pod of *Pongamia* were precultured for 10 days and 20 days in varying concentrations of TDZ in MS basal media and transferred to GR free MS basal media for induction of *de novo* organogenesis. Preculture of 20 days in 11.35 μM TDZ yielded more number of buds than 10 days culture. Proximal segment of the cotyledon demonstrated highest morphogenic potential. The best condition for optimum caulogenic response is, exposure to TDZ 11.35 μM for 20 days with abaxial side in contact of medium followed by elimination of TDZ from media to promote differentiation of these buds to elongated shoots. Plants were obtained by rooting of these shoots. *De novo* origin of the organogenic buds was confirmed using histological techniques. This is the first report on *in vitro* protocol of *Pongamia pinnata* via adventitious organogenesis (*Sujatha et al.*, communicated in *Trees-structure and Function*). The protocol may find application in studies on genetic transformation, isolation of somaclonal variants and in induction of mutants. It may also find application in study of the opposite roles of TDZ in induction and differentiation of buds. Embryo axis was tested as an alternative explant for induction of *de novo* organogenesis. TDZ induced adventitious buds did not differentiate into shoots on transfer to GR free MS media. Efforts taken to differentiate these buds are described.
Efforts made towards regeneration of *Pongamia* via somatic embryogenesis using different explants like cotyledon, embryo axis and immature seeds from green pods produced embryo like structures which did not differentiate further to generate plantlets.

The trees (ten numbers) used as source of explants were studied to assess the variation/uniformity. Variations were noted in morphological characters like pod shape and size, seed shape and size, and color. Oil content of the seeds showed variation ranging from 12 to 28%. For molecular analysis DNA was extracted from the tender leaves of these trees using Khanuja’s DNA extraction protocol which yielded good quality and quantity of DNA. Band patterns obtained from ISSRs were analysed using Jaccard’s coefficient to study the extent of similarity or variability among the selected trees. Highly polymorphic band pattern were obtained within the selected trees. ISSRs identified the 76% variation (polymorphism) among the ten trees tested.

Fidelity studies undertaken on the *in vitro* raised *Pongamia* plantlets produced no polymorphic band pattern with the primers used. Monomorphic pattern of *in vitro* raised clones of *Pongamia* validates the protocol developed for clonal propagation of *Pongamia*. The variation existing in this species emphasizes the importance of micropropagation techniques for cloning the superior genotypes. In this study ISSR-PCR techniques are applied in the germplasm characterization of wild and tissue cultured derived *Pongamia* plants (K.Sujatha et al., Manuscript under preparation).