7. Discussion

Ex situ conservation is one of the strategies for conservation of the “red listed” medicinal plants (Figure 1). Assessment of the genetic diversity of the conserved germplasm by RAPDs was considered as one of the easy and simple techniques where the amplification occurs across the total genome as these are random primers (Figure 2). RAPD technology has been applied to a number of medicinal and aromatic plants of conservation concern (Table 1). To date there are many markers investigated which have both advantages and limitations. RAPD technology is the most commonly applied tool, for investigating the molecular diversity of medicinal plants. (Table 2).

7.1 Collection and conservation of chosen species

*Pterocarpus santalinus* is an endemic and endangered tree species (Figure 6) in Deccan ecoregion (Figure 3). Its distribution is restricted to the Kadapa (Cuddapah), Nellore and Chittoor districts of A.P (Figure 4). Different accessions were collected from 27 different locations during the months of May - September (Table 6). The seeds were air dried and accessions in the form of seedlings and plants (300) were grown in experimental site and gene bank (Figures 8 and 9). *R. serpentina* and *R. tetraphylla* are distributed in different districts of A.P (Figure 5). The seedlings and seeds were collected during the months of January to December from different locations of A.P (Tables 7 and 8) and were conserved in the experimental field and seed bank (Figures. 10 and 11).
Among the various *ex situ* conservation methods, seed storage is the most convenient strategy for long-term conservation as seeds being perennating structures of plants, represent a condition of suspending animation of embryos, which is best suited for storage (Kameshwararao, 2004). According to the Harrington’s thumb rule of seed storage, lower the moisture level and temperature of stored seeds, greater is the storage life. Therefore, air dried seeds of all the collected accessions with low moisture level of 4 to 6% were placed in airtight plastic bags and stored in seed bank (Figures 9, 10 and 11). Their viability was checked periodically every six months.

The importance of morphological features and time of collection of raw material is reported to affect the stored seeds viability (Rawat and Uniyal, 1993). Pod characteristics like length, width and weight, which indicates the amount of seed reserve varied among different accessions of *P. santalinus* (Figures 23 and 24) (Arya et al., 1993). Apart from storage of seeds in seed bank, seedlings are also grown in experimental garden.

Several *in vitro* techniques have been developed for storage of vegetatively propagated and recalcitrant seed producing species. In general, they fall under two categories. Slow growth procedures where germplasm accessions are kept as sterile plant tissues or plantlets on nutrient media. This provides short and medium term storage options. Cryopreservation is another *in vitro* technique where plant material is stored in liquid nitrogen for long-term storage. Experiments conducted revealed that *in vitro* multiplication in case of *P. santalinus* was not satisfactory. However *in vitro* conservation has some advantages, as the cultures are not subjected to environmental disturbances (Withers and Engelmann, 1997).
In *R. serpentina* and *R. tetraphylla* attempts have been made for micropropagation. Hence in the present study only genetic diversity and similarity in between both the species was investigated.

### 7.2. Seed germination in *P. santalinus*

Knowledge of seed germination and seedling establishment is a prerequisite for the successful implementation of conservation activities especially for tree species like *P. santalinus* (endangered). Factors affecting seed germination in *P. santalinus* include seed dormancy, temperature, water stress, predation, seed size, light intensity, soil moisture, seasonal variations, different locations of collection etc., According to Kalimuthu and Lakshmanan (1995), *P. santalinus* inherently shows poor seed germination.

Temperature plays a key role in germination of *P. santalinus*. The optimum temperature for seed germination of *P. santalinus* found to be between 20 and 25°C in *P. santalinus* (Teketay and Granstorm, 1997). The rate of germination varied under different light regimes in most of the examined species collected from the dry forests. Differential concentrations of mineral nutrients in embryos and seed coats also influence seedling establishment, irrespective of seed size.

Low viability is usually due to loss of moisture, which is associated with loss of hair from the seed coat. Increased leachate conductivity and decreased fatty acid content due to aging in certain seeds are other reasons for loss of viability and decline in germination percentage (Thapliyal and Connor, 1997).

Competition from annual herbaceous flora is one of the limiting factors for *in vivo* seed germination of *P. santalinus*. Seed size represented a trade off between
seedling establishment and seed dispersal efficiency in wind-dispersed tree species like *P. santalinus*. It influences the dispersal and seed water relations, emergence, establishment, survival and growth of seedlings. Small seeds have a better chance to enter into the soil easily than larger seeds, and thus, facilitate the build up of persistent soil seed banks, crucial for regeneration of plant species. A greater food reserve in larger seeds may enhance its ability to persist by providing metabolic requirements during quiescence period, until the availability of suitable conditions of light or moisture, which stimulates germination, thereby enhancing seedling survival and growth (Milberg and Lamont 1997). The young seedlings from large seeds withdraw nutrients for their successful establishment, survival and early seedling growth. Significant pod variation was noted among the collected accessions (Figure 22).

Dormancy in recalcitrant seeds can be overcome by mechanical or acid scarification or sometimes by transit through animal guts. *P. santalinus* seeds are highly recalcitrant with a dormancy period of one year. In the undisturbed forests of Kadapa (Cuddapah) district of A.P, (Figure 6a), *P. santalinus* is the most dominant species. The seeds are bigger compared to seeds collected from Vishakapatnam (A.P, India) and require shade for survival of the seedlings. Seeds from different locations exhibited differences in germination and seedling growth. Thus, emergence, establishment and growth of seedlings face very heterogeneous situations even in the native territory i.e., in Kadapa district (Cuddapah) and that of Veligonda Hills (Nellore District).
During *in vivo* seed germination many treatments have been used to break the dormancy. Acid, hot water and mechanical scarification have been applied to be found suitable in a majority of forestry species. In *P. santalinus*, mechanical scarification was found to be more effective. Such findings were also reported in *Olea europaea* and *Podocarpus falacatus* by Teketay and Granstorm (1997). Pods of *P. santalinus* collected from various areas responded only to potassium nitrate (100 ppm) treatment (dormancy breaking agent) but the percentage of germination was very less (2%). Dormant seeds suppress the negative demographic effect of reproductive failure and permit the species to avoid environmental conditions potentially unfavorable for seedling establishment. A hard seed coat prevents entry of moisture during isolated showers in the middle of a long dry season while permitting the same during a sustained rainy season. Dormant seeds generally remain viable for long periods of time as in the case of seeds of *P. santalinus*. The extent of dormancy varies within a species, and as a result, individual seeds become permeable to water at different times, which results in staggered seedling recruitment providing an insurance against spells of unfavorable conditions. Thus the soil seed bank produces seedlings continuously during permeable conditions for several years due to different periods of dormancy.

*In vivo* seed germination studies in *P. santalinus* showed maximum seed germination in pods collected from Balpally (Kadapa District, A.P) when compared to other locations (Table 9 and Figures 12 a and b). The larger size of seeds and high germination rate compared to rest of the accessions place them under desirable and elite accessions.
In vitro experiments using Balpally (Kadapa District) seeds on MS (liquid), ½ MS (liquid), 3% sucrose, 1% agar and MS with 0.1% phytogel resulted in 100% seed germination. With 3% sucrose being the most cost effective (Table 10, Figure 13) and successful when cultured with the micropylar end touching the medium facilitating nutrient uptake from the medium. Thus, in vitro seed germination proved to be very useful for enhancing seed germination to build up seedling stocks.

7.3. In vitro plant regeneration

In vitro propagation of trees using tissue and organ culture has clearly proved to be a useful technique for multiplication of a number of forestry tree species. Direct organogenesis seems to be a desirable method since there is no callus phase involved in the shoots obtained and hence reducing the chance for induced variations. Forest trees in general, and legumes in particular are recalcitrant. Even though there are a few reports on organogenesis and micropropagation of tree legumes like Acacia sps. (Aradhana et al., 1989), Albizia sps. (Tomar and Gupta, 1988), Dalbergia sissoo (Suwai et al., 1988) Sesbania sp. (Khattar and Mohanram, 1983) etc., regeneration has been mainly from seedling explants which is not desirable from the tree improvement point of view due to chances for variability and lower multiplication rate. Efficient regeneration is a prerequisite for genetic manipulation and transformation studies using different explants.

In nature, P. santalinus reproduces via seeds, but the low percentage of germination limits its propagation as it requires a long stratification period of about one year to break the dormancy partly by weathering process or by microorganisms (Arockiasamy et al., 2000). Hence, an alternative method like in vitro propagation
was checked to understand whether the plant can be propagated true to its type or not. Among various explants used like nodal segments, shoot tips, leaves, cotyledonary nodal meristem, hypocotyls and seeds for multiple shoot regeneration, only whole seeds showed excellent response (Table 20). The explants used were collected from the plantations of UH campus and also from the in vitro regenerated seedlings (avoid problems of contamination). Varied morphogenetic responses were observed with different explants (Table 13).

7.3.1. Role of media, sucrose, agar and orientation

Initially nodal segment explants were cultured on different types of media and no significant difference was found (Table 11). Hence, further experiments were carried out on MS medium which is the most universally accepted standard medium amenable for good and efficient growth of multiple shoots. Different morphogenetic responses were observed when various plant growth regulators at different concentrations in MS medium were used for screening (Table 12). Sucrose at 3% was found to be optimum for multiple shoot production possibly because higher concentrations of sucrose (4 and 5%) may increase the levels of polyphenols, which results in browning of cultures and subsequent growth inhibition.

For different explants used i.e., nodal segments, leaves, seeds, shoot and cotyledonary meristems, of all the different percentages of agar tried, 0.6 % (Table 14) agar was found to be most effective for multiple shoot regeneration due to the semisolid nature of the agar which allows profuse proliferation of shoots when compared to 0.8% agar.
Besides explant type (seeds better compared to others), orientation of the explant in the cultures plays a significant role in induction and proliferation of multiple shoots (Polisetty et al., 1977). It was observed that horizontal orientation of the explant was more effective (Table 15) when compared to vertical orientation because horizontal orientation results in the whole surface of the explant in contact with the semisolid medium. Profuse sprouting was observed from all the exposed surfaces of the explant profusely (Figure 14a) which was later transferred onto medium with similar hormonal composition in vertical orientation (Figure 14 b), for proper growth and maturation of plantlets. Thus as observed in Chickpea (Suhasini et al., 1996), orientation of the explant has a significant effect in *P. santalinus* which was further modified by other factors like age of the explant and season of collection.

A 2-day-old seedling was placed with micropylar end of the seed downward into the medium, did not produce multiple shoots initially. On the other hand, 3-4 days old seedling explants when used in a similar way, numerous multiple shoots were produced. Thus different age and orientation of the explant caused differences in multiple shoot regeneration and can be attributed to increased in time for starch accumulation with BAP treatment resulting in multiple shoots. Heavy and early accumulation of starch in BAP treated explants was related to induction of shoot primordia. Hence, it clearly supports the view that orientation plays a major role in multiple shoots production, which in turn is related to starch accumulation. Breakdown of starch into sugars by alpha amylase had negative influence on the process of differentiation. Thus, treating the explant with BAP resulted in accumulation of starch with a corresponding reduction in solubilization of starch by
alpha amylase, which might be linked with multiple shoot production (Thorpe and Murashige, 1970).

7.3.2. Nodal segments as explants

Induction of adventitious shoot buds in BAP treated explants by suppressing the apical dominance was reported by Polisetty et al., 1997. Successful shoot development or organogenesis was observed with utilization of nodal segment explant with axillary buds (one per node) though multiple shoots formed were very less compared to seed explants. Around 80% of these explants developed actively growing buds, mostly 6-7 shoots per nodal segment after 30 days of culture on MS medium fortified with different cytokinins either singly or in combination. Earlier and higher frequencies of bud break, as well as varying degrees of multiple shoot formation occurred on BAP supplemented medium compared to the control medium without any hormones. In nature, these axillary buds may remain dormant for various periods depending on the growth pattern and environmental conditions, however by culturing the nodal segments on medium containing appropriate concentrations of cytokinins, it is possible to break the dormancy and subsequently enhance development of multiple shoots (Figure 15).

The growth and multiplication of axillary buds was greatly influenced by the season of explants collection. Sprouting was better when they were collected during the months of September to December. During rainy season axillary buds were free from phenolic exudates and resilience is broken and proliferated into multiple shoots. At higher and lower concentrations of cytokinins either singly or in combinations, initiation of callus was greater. Swelling of the dormant axillary bud within a week
followed by differentiation into two to three shoot buds in three weeks was observed along with development of callus in different concentrations of KN and BAP. After 5 weeks maximum number of shoots observed were 6.5, 5.0, 4.8 and 6.0 respectively on MS with 3.0 mgL⁻¹ BAP, MS with 3.0 mgL⁻¹ KN, MS with 2.0 mgL⁻¹ TDZ and MS with 0.1mgL⁻¹ BAP + 3.0 mgL⁻¹ TDZ (Figure 15).

Superiority of BAP over KN was demonstrated by several workers (Rech and Pires, 1986; Kukreja et al., 1991; Vaneck and Kitto, 1990; Mishra and Bhatnagar, 1995). This may also indicate that these explants (seeds and nodal segments) contain sufficient endogenous level of auxins or are capable of its de novo synthesis which can induce shoot formation even in a medium containing cytokinin alone (Julliard et al., 1992).

Nodal explants were more responsive than apical shoot meristems and leaf explants, as multiple shoot production was observed with nodal segments, whereas development of callus was observed with shoot meristems and leaf explants. This differential morphogenetic response could be due to differences between the physiological states of the buds on different regions of the stem when compared to that of other explants (Table 13) (Vieitez et al., 1985). Similar results were also reported in Syzygium cumini and Morus australis (Yadav et al., 1990). The less frequent shoot initiation was preceded by callusing in case of apical meristems and leaf explants. Hence, shoot tip cultures and micropropagation using leaves was less desirable when compared to seeds, as the number of regenerated shoots was very few in number. Nodal explants can be used for micropropagation in case of unavailability
of seed explants though the number of regenerated shoots are less compared to seeds (but better than shoot tip and leaf explants).

When cotyledonal nodal meristems were used as explants, multiple shoots (12.0) were obtained on MS medium with 1.0 mgL\(^{-1}\) BAP (Figure 14 e) but there was no further elongation of shoots which became stunted. Even when transferred onto the shoot elongation medium like MS with different concentrations of GA\(_3\) (0.1-7mgL\(^{-1}\)), no elongation was noticed and may be due to some of the external conditions or the inability of the shoots to further absorb the plant growth regulators from the medium and thus shoots remained inert. Hence, the usage of cotyledonal nodal meristem was not continued further.

All the explants at the initial stage increased in size and became green in colour. In most of the cases callogenesis and adventitious bud initiation took place simultaneously as seen in the case of nodal segments and leaves (callus). The degree of bud induction and callus proliferation varied greatly with the growth regulator composition of the medium. No correlation was observed between the amount of callus and number of shoot buds per culture. The axillary buds at the initial stage appeared as tiny tube like structures that resembled bulbets seen in some of the tuberous crops. The size of the initial explants i.e., nodal segment, hypocotyls and shoot tip is significant for multiple shoot regeneration.

The observed shoot forming ability of all the in vitro derived explant types such as nodes, meristem, leaves, cotyledonary nodal meristem, hypocotyls did not influence the rate of proliferation as compared to explants tested from the field grown plants. Repeated subculturing of nodal segments, leaves, and seeds from seed cultures
helped to achieve continuous production of callus free, healthy shoots at least through five subculture cycles. A similar phenomenon was also observed in *Morus australis* (Pattnaik *et al*., 1996). From single seed, 80% of the shoots showed good rooting on MS medium without exogenous hormones. The results suggested that seeds when used as explants, a threshold level of endogenous growth regulators accumulated during culture initiation, which enabled the explants to develop optimum number of multiple shoots initially in the MS basal medium, and at reduced levels of BAP and KN without auxin augmentation. Hence, endogenous hormonal level plays an important role in shoot multiplication.

### 7.3.3. Seeds as explants

Among all the explants tried seeds, of *P. santalinus* responded most favourably in the presence of BAP and KN for multiple shoot induction (Figures 15-18) as in *Albizzia chinensis*, a tree species (Sinha *et al*., 2000). Patri *et al*., (1988) have reported shoots with scaly leaves from cotyledonary node. Arockiasamy *et al* (2000) reported the influence of growth regulators and explant type on *in vitro* shoot propagation, but in all these cases, the number of multiple shoots regenerated were very less when compared to the present study. Seeds from Balpally (Kadapa District) produced 2-3 shoots when cultured on MS basal medium unlike seeds from other locations which showed normal seed germination and were hence used for further comparision with other explants. TDZ, a urea-derived potent cytokinin for woody plant tissue culture was extensively used for the induction of shoot regeneration in several plant species (Huetteman and Preece, 1993; Li *et al*, 2000; Liu *et al*., 2003). However it had no effect in *P. santalinus*. 
During initial subculturing, the mother explant was kept intact with proliferated shoots. Later increasing or decreasing concentration of hormones resulted in decreased rate of shoot regeneration. When the explant was treated with BAP there was a significant increase in phenol content. Thus increasing the concentration of BAP to a higher level leads to decrease in the number of multiple shoots. Hence changes in secondary metabolites like phenols seems to play an important role in determining the BAP induced multiple shoot differentiation. An adverse effect of phenols on differentiation was reported by Bhat and Chandel (1991). At the same time maintenance of auxin and cytokinin, ratio was found to be necessary for differentiation.

When a single node obtained from the multiple shoots was cultured onto MS media with 1mgL⁻¹ BAP 3-4 multiple shoots were produced (Figure 19c) which were further used for rooting. Thus among all of the explants tried seeds responded most favourably in the presence of BAP and KN in *P. santalinus*. This could be due to many factors prominent among which is that the seeds collected from Balpally (Kadapa District) may be variants and further investigation of the multiple shoots needs to be done to determine their origin (polyembryony or apomixes) (Table 20)

The requirement of embryonic axis along with cotyledons for inducing multiple shoots was very important. Evidently the presence of cotyledons was essential for maximum shoot production potential. The regeneration of multiple shoots from seeds collected from Balpally (Kadapa District) may be due to the phenomenon of either polyembryony; apomixes or due to variations, which has to be further worked out in future (Table 20)
Although several reports on tissue culture of many plants are available, information on plant losses during in vitro culture caused by microbial contamination is rather scanty. In *Colocasia esculenta* (Taro) it was found that microorganisms can live within plant tissues for longer periods in vitro without being pathogenic and show up in cultures during short environmental changes which may inhibit growth rate and decrease the potential of in vitro propagation. This was also a prominent observation in seeds of *P. santalinus*, which were collected from forests of different areas. Contaminants could become pathogenic in vivo when the plants are introduced into another climate. Furthermore, metabolites of the contaminants can be toxic to the culture during short climatic changes in the growth room. Leifert *et al* (1991) mentioned that microbial contaminants may lead to heavy loss of plants.

Deleterious pathogenic microorganisms, which exist endogenously, are activated during culture conditions. In case of Taro (*Colocasia esculenta*), high death rate of explants was observed in tissue culture due to the presence of casual endogenous microorganisms (Gunua, 1921). Initially 50% of the seeds of *P. santalinus* were contaminated with bacteria and fungi which were endogenously associated. Contaminated cultures were able to change the colour of the growth medium from colourless to yellow and were able to survive after treatment with an antibiotic (Streptomycin) or (0.1 % Bavastin) fungicide followed by transfer to fresh medium.
7.3.4. Rooting in *P. santalinus*

Rooting was found to be difficult in *P. santalinus*. Auxin in the medium generally promotes rooting, while in the present study, auxins in MS medium were ineffective in rooting of individual shoots. Rooting was observed only when the shoots were not separated and left in clumps. Thus fully grown plantlets with 30-40 expanded leaves and well developed roots in bunches were transferred into magenta boxes successfully (Figure 19 a, b and Figure 20). The survival rate was only 20% when the plantlets in soilrite were transferred to glass house.

The role of activated charcoal in a nutrient medium for tissue culture was discussed by Misson *et al.*, 1982. Charcoal is thought to remove the inhibitory material that may be present in the medium that originate from the explant itself. Anagnostakis, 1974 and Fridborg *et al* 1978 found that compounds excreted from growing cells of *Daucus* and *Allium* could be adsorbed by activated charcoal to allow embryogenesis and root formation that did not occur in cultures lacking activated charcoal. Stenitz and Yahel (1982) found a need for activated charcoal in the medium for the production of bulbets of *Narcissus tazetta*. Activated charcoal incorporated into the media for our study, had no effect. The percentage survival observed in *P. santalinus* was very less (20%).

Hence, micropropagation has been advocated as one of the most viable biotechnological tools for *ex situ* conservation of germplasm. Similar reports are plenty and many of the groups have been successful in the micropropagation of number of plants maintaining genetic stability of the tissue cultured clones. (Gangopadhyay *et al.*, 2003; Ramalakshmidutta *et al.*, 2003) but whereas in case of
P. santalinus micropropagation technique was not so effective in terms of conservation management purposes since the percentage survival of plants in field conditions after transfer was very less due to many factors one of them being the highly recalcitrant nature of the species (20%) during hardening. Further studies need to be planned to emphasize on identification, description, documentation, and to find out the relationship of these microorganisms with P. santalinus. Hence both in vivo and in vitro seed germination would be the most feasible.

7.4. Morphological variations in P. santalinus

The morphological variations are not significantly correlated with the geographical distances. Morphological data indicated considerable phenotypic variations among various accessions of P. santalinus (Figure 22).

The study of natural variations has proved to be useful for analyzing the genetic basis of some developmental processes in the model system Arabidopsis thaliana (Perez-Perez et al., 2002). The large phenotypic variability obtained for the quantitative traits facilitated a clear distinction among the sixteen accessions collected from different geographical locations indicating the existence of region specific adaptations due to the influence of environmental and edaphic factors among all the locations. The accessions of P. santalinus showed large differences same could be true here for the large differences observed in pod weight between Araku (Vishakapatnam District) and Papavinasanam (Chittoor District) pods, pod length between pods of Balpally (Kadapa (Cuddapah) District) and Vishakapatnam, pod width between Papavinasanam (Chittoor District) and Rapur (Nellore District) pods, number of shoots between Rapur and Sanipaya (Kadapa (Cuddapah) District) plants,
number of nodes between Rajamundry (E. Godavari District) and Sorakaipalem (Chittoor District) plants and leaf length and width between plants from Sorakaipalem (Chittoor District) and Papireddypally (Kadapa (Cuddapah) District) (Figures 23-27). The conventional morphological markers used for characterization of genotypes cannot be relied upon as their expression is influenced by environmental factors and developmental changes.

Plant populations under different environmental selection pressures generally show phenotypic differences. Such phenotypic differences are due to genetic diversity. The high levels of genetic diversity as observed in case of accessions of *P. santalinus* also accounts for the high levels of allelic diversity.

Generally qualitative traits reveal less genetic diversity than quantitative traits. The available moisture of the growing environment is very important for plant growth. Phenotypic traits are controlled by genes and affected by environment. In *P. santalinus* the phenotypic data also revealed polymorphism which indicate genetic variation. Therefore, phenotypic traits further strengthen the occurrence of molecular diversity (Perry and McIntosh, 1991).

7.5. **RAPD analysis in *P. santalinus*, *R. serpentina* and *R. tetraphylla**

RAPD markers have been employed as an alternative for morphological and biochemical markers. (Dawson *et al.*, 1993; Pei *et al.*, 1995; Su *et al.*, 1999; Wolfe and Liston; 1998, Yoon and Glawe, 1993; Esselman *et al.*, 2000).

The data from RAPDs do not depend on strict dominant and recessive allelic frequency. Estimating the genetic differentiation coefficient among populations using RAPDs has been problematic due to their dominance, and analytical methods usually
rely on knowledge of the selfing rate or assume Hardy-Weinberg equilibrium (Lynch and Milligan, 1994). This assumption does not hold when populations exhibit fixed heterozygosity, hence in our studies an alternative method i.e., Dice coefficient was used to partition the genetic diversity which is supposed to be in accordance with the RAPD data and it was found consistent in showing the variation within accessions. DNA based polymorphism contributes towards assessing phylogenetic relationships among different species and genera. The tissue age, pathogen infestation, intra population contamination and PCR conditions are reported to introduce some levels of error in RAPD analysis. Therefore DNA from young uninfected plant tissue, is the best suited material to achieve consistent results (Staub et al., 1996).

One of the objective of the study is to find out the genetic distance between different accessions of *P. santalinus*, *R. serpentina* and *R. tetraphylla* individually and genetic similarity in between *R. serpentina* and *R. tetraphylla*. *P. santalinus* is a woody plant species where the leaves are exceptionally rich in of polysaccharides, polyphenols, tannins, hydrocolloids (sugars and carragenans), and other secondary metabolites such as alkaloids, flavanoids, phenols, terpenes and quinines which have interfered with the DNA isolation and further experiments in molecular technology. Polysaccharides interfere with the PCR by inhibiting *Taq* polymerase activity (Fang et al., 1992) which inturn can inhibit RAPD reactions. Polysaccharides like contaminants, which are undetectable by most criteria, can cause anomalous reassociation kinetics but polysaccharide co-precipitation is avoided by adding a selective precipitant of nucleic acids, i.e., CTAB to keep polysaccharides in solution.
The presence of polyphenols, which are powerful oxidizing agents present in many plant species, can reduce the yield and purity of DNA by binding covalently making it useless for most research applications (Katterman and Shattuck 1983; Peterson et al., 1997; Loomis, 1974). Additionally tannins, terpenes and resins are difficult to separate from DNA (Doyle and Doyle, 1987, Ziegenhagen and Scholz, 1998).

Therefore the problems encountered in the isolation and purification of DNA specially from medicinal and aromatic plants include degradation of DNA due to endonucleases, coisolation of highly viscous polysaccharides, inhibitor compounds like polyphenols and other secondary metabolites which directly or indirectly interfere with the enzymatic reactions and moreover the contaminating RNA that precipitates along with DNA causes many problems including suppression of PCR amplification (Pikkart and Villeponteau, 1993), interference with DNA amplification involving random primers, e.g. RAPD analysis and improper priming of DNA templates during thermal cycle sequencing (Mejjad et al., 1994; Yoon and Glawe, 1993).

These factors do not permit optimal DNA yields from one isolation protocol, and perhaps even closely related species may require different isolation protocols (Weishing et al., 1995). Hence *P. santalinus* DNA was isolated by using Plant DNA Zol isolation Kit where a good quality and quantity of DNA was obtained which was used for RAPD reactions (Figure 28). In case of *R. serpentina* and *R. tetraphylla* DNA isolation was comparatively easier with reference to that of *P. santalinus* and
pure DNA was obtained following the established CTAB method with few modifications (Figures 55 and 56).

The differences among accessions of *P. santalinus*, *R. serpentina* and *R. tetraphylla*, collected from different locations, could partly be explained as a result of both abiotic (geographical, e.g., hydrographic connections, or climactic differentiation, e.g., annual rainfall differences) and biotic (pollination between populations and seed dispersal etc) factors. It is expected that obligate outcrossing species show more genetic variation at the population level (Apostol *et al.*, 1996; Cardoso *et al.*, 1998) as observed in *P. santalinus*, *R. serpentina* and *R. tetraphylla*.

For a species with limited gene flow and over 50% variation among populations, it is necessary to collect samples from at least six locations (based on which accession no. is given) in order to conserve 95% of the genetic diversity of the species. Hence, our minimum size of the accessions was at least six to minimize the external effects. For a species with only 20% variation among populations, samples taken from two populations are enough to get the same results as above. (Pei *et al.*, 1995).

With all the tested 40 primers genetic polymorphism was 100% in *P. santalinus*, whereas in *R. serpentina* and *R. tetraphylla* it was 70% and 50% respectively. Genetic similarity between *R. serpentina* and *R. tetraphylla* was found to be 85%, exhibiting a higher genetic diversity among the collected accessions (Tables 23, 24, 29, 32, 34 and 35, Figures 30-53 and Figures. 58-97).

Among the forty primers tested, *P. santalinus* showed 100% polymorphism with 26 primers (Table 22) indicating higher genetic diversity within populations of
*P. santalinus.* Distinct polymorphic bands have been observed on 2% agarose gels (Figures 29-52) (Hamrick et al., 1992) *R. serpentina* showed 100% polymorphism with 16 primers and in *R. tetraphylla* only with 3 primers (Figures 57-96, Tables 28, 31 and 33). In case of accessions of *R. serpentina* the genetic variation is more when compared to that of *R. tetraphylla* but both the species show a genetic similarity of around 85%. RAPD data suggests that in woody legumes most of the variation is maintained within the populations (Schierenbeck et al., 1997). Similar results are reported in tropical in similar to the genetic variation of a tropical tree legume, *Gliricidia sepium* which showed more than 60% genetic variation (Chalmers et al., 1992).

The high levels of variation found within different accessions of chosen species suggests that sampling from a few localities for either breeding or conservation could capture a large proportion of the variation within the species. The genetic diversity can be explained by the aid of calculation of polymorphism levels and cluster diagram.

The mean level of genetic diversity within 15 accessions of *P. santalinus* is 3.168. The range of genetic diversity calculated in terms of genetic distance is 0.14 - 0.76 (Figure 53). Similarly the mean level of genetic diversity among accessions of *R. serpentina* is 3.168 and that of *R. tetraphylla* is 2.733 and when both of the species are analyzed it is 3.940. The range of genetic diversity calculated in terms of genetic distance for *R. serpentina* is 0.596-0.928 (Figure 97). *R. tetraphylla* it is 0.816-0.932 (Figure 99) and for both of them it is 0.407-0.955 (Figure 101). From this it is evident that the accessions from different geographical locations exhibited a wide range of
genetic distance, which did not show any correlation with geographical distances between the collection sites, negating a simple isolation by physical distance.

In *P. santalinus*, cluster analysis based on Dice coefficient showed two major groups (Figure 54) indicating that in cross pollinated plants, high levels of differentiation among populations and relatively less within-population genetic variation exists. The dendrogram obtained by the aid of similarity matrix revealed that there is a similarity of 76% between the accessions collected from Kerala, India and Raichoti (Kadapa District) which clearly depicts that genetically they are similar which was confirmed from the owner of the nursery that they were collected from A.P and were grown in Kerala as plantations. There is also a close similarity of 76% observed between the accessions collected from Talakona (Chittoor District, A.P) and Gadela (Kadapa District, A.P) though geographically they are distantly placed in contrary. The accessions collected from Tirupathi (Chittoor District, A.P) and Narsingapuram (Chittoor district, A.P) though closely placed geographically, their a genetic similarity of only 32%, which clearly indicates that there is no correlation between genetic make up and geographical distances.

The pattern of genetic diversity in *P. santalinus* may be maintained due to effective gene flow within populations. Animal drops which aid in seed dispersal may also contribute for inducing variations indirectly within the populations thus accounting for the high levels of genetic variation (Loveless and Hamrick, 1984; Hamrick and Godt, 1989). Distribution range and population size have been identified as the major correlates within population genetic variation in tropical tree species with restricted populations showing significantly less variation than those with
broader distribution (Loveless, 1992; Travis et al., 1996). In case of *P. santalinus*, inspite of its smaller population size and being endemic in certain districts of A.P i.e., Kadapa (Cuddapah), Chittoor, Nellore and Kurnool, high genetic variation is observed, which might be due to highly cross pollinated nature of the plant. It was reported that outcrossed wind pollinated species exhibit vast variation within populations (Loveless, 1992; Loveless and Hamrick, 1984)

In case of *R. serpentina* and *R. tetraphylla*, when the RAPD data was analysed comparitively two distinct groups were observed (Figure 102). In the dendrogram of *R. serpentina* the accessions collected from forests in Sukumamidi (East Godavari,A.P) showed 62% genetic similarity with other accessions collected from various locations and falls in a separate cluster. Whereas accessions from Araku (Vishakapatnam, A.P) and Hyderabad (A.P) show a similarity of 95% though they are very distant geographically. Similarly the accessions of *R. tetraphylla* collected from Dulapally (Medak District, A.P) and Vijayawada (A.P) show a genetic similarity of 93% though they are quite distant geographically. This situation arises only in the case of natural populations where there is a free/random pollen flow and fertilization, as is the case of the cross-pollinated species.

The grouping of these populations is independent of the geographical distance. Study of interspecific variations and assessment of the genetic similarity among populations of *R. serpentina* and *R. tetraphylla* showed that the 13 populations were divided into two distinct groups based on the difference at species level as evidenced by the dendrogram. Polymorphism of 90 % was observed among populations of *R. serpentina* and *R. tetraphylla* in interspecific diversity analysis.
There is 85% genetic similarity between \textit{R. serpentina} and \textit{R. tetraphylla} by cluster analysis in Unweighted Pairwise Group Matrix for Arithmetic Average (UPGMA).

\textit{R. tetraphylla} which is a common plant, can be useful for genetic improvement of \textit{R. serpentina}.

The analysis of genetic similarity and dissimilarity in terms of similarity matrix and cluster analysis i.e., genetic diversity of \textit{R. serpentina} and \textit{R. tetraphylla} species by using limited set of primers proved to be promising for further investigation. It proved that the accessions collected from different locations showed similar morphologies (leaf morphology, leaf length, colour of the petiole, colour of the midrib, flower morphology, fruit morphology), their RAPD fingerprinting differed markedly. On the other hand morphological differences were observed between species in leaf, flower and fruits, hence both genetic similarity and diversity between both the species of \textit{Rauvolfia} was noticed.

Therefore, analysis of RAPD data could be useful to detect genetic differentiation as well as similarity between accessions of \textit{R. serpentina} as well as \textit{R. tetraphylla}. A close phylogenetic proximity between \textit{R. serpentina} and \textit{R. tetraphylla} was shown as per the dendrogram. RAPD marker provides equivalent levels of resolutions for determining genetic relationships (Santo et al., 1994). Reliability of RAPDs among closely related taxa and the limitation of RAPD data for producing expected associations among more divergent taxa was observed in \textit{Pisum} species (Hoey et al., 1996). Similarity between \textit{R. serpentina} and \textit{R. tetraphylla}, may be in terms of some of the morphological or genetic traits and same is the case with
diversity. To analyze in detail further work has to be done so that the appropriate reasons for both the similarity and diversity between both the species can be unraveled.

The significant variations in the accessions of *P. santalinus* collected from Tirupathi (Chittoor District, A.P) when compared to other accessions from various locations need to be investigated further. Similar is the case with one of the accession of *R. serpentina* collected from Sukumamidi (East Godavari, A.P). Such observations have been reported previously in *Hordeum spontaneum* populations by Dawson *et al.*, (1993). It can be inferred that in the accessions, which are clustered in similar groups, there is an effective gene flow in those areas but whereas with the accession of *P. santalinus* collected from Tirupathi and *R. serpentina* collected from Sukumamidi, the gene flow is less hence, they are highly divergent when compared to other accessions (Figures 56 and 99). This may be due to highly cross-pollinated nature of the plant and due to the occurrence of some mutations and rearrangements in the genome, resulting in variation. In *P. santalinus* some of the morphological parameters like pod weight, pod length and leaf length were in accordance with that of the molecular data but it may not represent the exact trait as there may be many other phenotypic traits which may exhibit variation.

Genetic variation decreases with decrease in population size (Mosseler *et al.*, 1992; Baskauf *et al.*, 1994; Gray, 1995; Kappe *et al.*, 1995; Frankham, 1997; Palacious and Gonzalez-Candelas, 1997). One would therefore expect rare and endemic species of small population size, often associated with increased inbreeding and genetic drift, processes that lead to loss of genetic variation (Ellstrand and Elam,
Inbreeding is avoided in all the accessions of *P. santalinus* because the plants are dioecious, although within-population gene exchange is unavoidable. This situation may arise in natural populations where there is a possibility of free/random pollen flow and fertilization as in the case of most of the cross pollinated species or may be attributed to the formation of hybrids due to introgressive hybridization. Mutations may also play an important role in causing variations. In *P. santalinus* and in possibly other species which are rare and endemic high levels of genetic variation is maintained the reason for which it is not clear which is not yet clear. If a population has always occurred in small numbers, it means that it is adapted to that local conditions (Milligan *et al.*, 1994).

Sources of polymorphisms in RAPD assay may include base change within priming site sequence, deletions of priming site, insertions that render priming sites too distant to support amplification, and deletions or insertions that change the size of a DNA fragment without preventing its amplification (Williams *et al.*, 1990). In addition the polymorphisms of RAPD markers were observed as different sized DNA fragments from amplification. In *P. santalinus* the strict out crossing results in higher levels of heterozygosity (Wolff *et al.*, 1994).

The differences found among the dendrograms generated by RAPDs could be partially explained by different number of PCR products analyzed reinforcing the number of loci and their coverage of the overall genome, in obtaining reliable estimates of genetic relationships among the accessions of *P. santalinus; R. serpentina* and *R. tetraphylla* (Figures 54, 98, 100 and 102).
Another explanation could be low reproducibility of RAPDs (Karp et al., 1997). The putatively similar bands originating for RAPDs in different accessions are not necessarily homologous although they share the same size in base pairs. This situation may lead to erroneous results when calculating genetic relationships. Problems of the reliability and repeatability of RAPD markers are well known (Ellsworth et al., 1993). However in our experiments, high reproducibility with PCR products for RAPDs was observed.

The gene flow in higher plants is accomplished by dispersal of seeds and pollen as well as by vegetative mobility (Handel, 1985; Parker and Hamrick, 1992). Gene flow by pollen dispersal is often low in herbaceous plants (Widen and Swenson, 1992). In *P. santalinus, R. serpentina and R. tetraphylla* none of the accessions collected for our study have less than 15 Km distance to each other. Hence, the genetic structure of any of these accessions is stable and free from any gene flow into them. Hence there is a wide range of genetic differentiation. The genetic variation is related to the distances of pollen and seed dispersals. The seeds of *P. santalinus* are winged which favours the seed dispersal over long distances. *P. santalinus* species is bee pollinated and hence there are more chances of pollen dispersal resulting in a broad range of variations. A detailed on all the aspects related to variations is warranted.

Genetic drift over thousands of generations would lead to significant divergence. This trend may be reinforced for adaptive traits by selection of important ecological differences existing among the areas from where the accessions were sampled. Results from RAPD analysis indicates that genetic drift might have
occurred among the studied accessions of *P. santalinus*, *R. serpentina* and *R. tetraphylla* thereby producing population differentiation. The main reason being an overexploitation leading to shrinkage of their habitat. With a larger area of population, the probability of crossing among the individuals increases, which results in the retention of genetic variation. Though many individuals of these species were reported earlier in due course of time they have disappeared gradually along with environmental changes in their habitat. For decades much attention has focused on the genetic risks associated with small population size, not only from inbreeding and genetic drift, but also from gene flow. Until now, a precise empirical assessment of how well diversity has been characterized is unavailable (Ellstrand and Elam, 1993).

The wide range of variation observed among selected species may also be due to two evolutionary forces like pollen flow and local selection pressures. Pollen can be dispersed over large distances; this long-term reciprocal movement of pollen must also have contributed to the variation. Recent experiments using pollen traps have shown that oak pollen can migrate at several kilometers (Lahtinen *et al.*, 1996).

The local selection pressures may be due to the effects of environmental factors and due to struggle for existence in nature. The wide spread occurrence of the wind pollination and breeding systems that promotes outcrossing may lead to higher genetic diversity. Palynological and anthropogenic influences may also be attributed to high levels of genetic variation.
7.6. Intrapopulation variation in *R. serpentina* collected from Dulapally

Intrapopulation diversity in *R. serpentina* among eight plants collected from a nursery maintained by Forest Department of A.P, in Dulapally (Medak District) (Figure. 103) showed that one of the plant was highly variant and was falling into an entirely different group, it may be due to the highly crosspollinated nature of the particular plant and also some internal rearrangements occurring in the genome. The monomorphism exhibited by different plants collected from the same location indicates the occurrence of self-pollination in all the plants where homogeneity is being maintained. When intrapopulation variation analysis was carried out in one of the accessions of *R. serpentina* collected from Dulapally it was found that a very low level of genetic variation was found, presumably as a consequence of the techniques applied to seed production, responsible for genetic drift (Figures 104-115) (Tables 36 and 37). This stresses the need to address breeders to apply appropriate techniques for seed sampling. It also underlines the need for further monitoring of the genetic and demographic status of populations, if they decrease too much in size, they will become critically stochastic events (Lanteri *et al.*, 2003)