Discussion
DISCUSSION

Micropropagation has emerged as a tool for conservation of various endangered and endemic medicinal plants and is manifest with enormous reports on regeneration of such species (Stanilowa, et al., 1994, Wawrosch, et al., 2001). In vitro protocols for fast multiplication of endangered species could be very useful for taxa that are difficult to propagate through conventional means. Contamination and necrosis hampered during establishment phase in plant tissue culture might be possibly due to the under or over exposure of the explants to sterilants and the use of unsuitable types and concentrations of sterilizing agents. An appropriate explant sterilization procedure which was standardized by several trial and error methods could minimize the microbial contamination rate without harm to the tissue. The present result denoted that bacterial contamination was more frequent than fungal ones in all explants. This is probably caused by endogenous pathogens associated with various parts of the plant. Similar observations were made in Piper nigrum (Philip et al., 1992).

An essential step in establishment of cultures from juvenile explants is aseptic seedling germination, since the explants derived from aseptic seedlings form less phenol in the culture and are also highly morphogenic in nature (Ivano, et al., 1997; Saxena, et al., 1997). Use of Hormone free medium (BM) for raising aseptic seedling was the usual procedure adapted (Dawan, et al., 1992, Raharjo and Punja, 1993). In the present investigation seeds of P. tirumatiensis did not germinate in MS medium free of any growth regulators. However, in presence of GA₃ and BA seed germination was observed. The dormancy breaking effect of GA₃ was already reported (Kesera and Sen, 1987; Kulkarni, et al, 1993).
Tuber formation in vitro due to accumulation of starch in the root or stem is a natural physiological activity in any root and tuber crop. But in an in vitro system, this does not happen unless preconditioned by media or by incubation. This phenomenon was first studied in in vitro cultures of potato and was found to depend on factors like genotype, media components like sucrose, cytokinin and on day-length (Abbott and Belcher, 1986). In the present investigation tuberization occurred in seedlings grown on MS medium and was found to be influenced by sucrose and kinetin levels in the medium. Plant growth regulators have already been reported to influence in vitro tuber formation (Nakatami, 1994). In the present investigation, high concentration of sucrose (100%) induced maximum tuber formation in vitro under 16hr day length, which was similar to the results obtained by Ng et al., (1990).

MICROPROPAGATION

Clonal propagation has been successfully carried out using various explants like axillary and shoot tips (Venkatraman and Ravishankar, 1986; Kataeva and Popowich, 1993). The mechanism of vegetative propagation center around the formation and multiplication of shoot meristems, each being a potential plant. Higher plants have an indeterminate mode of growth in which the leaf axils contain subsidiary meristems each of which is capable of growing into a shoot that is identical to the main axis. According to the degree of branching that is characteristic of any species, only a limited number of axillary meristems develop, the majority being inhibited by apical dominance.

The primary leaves that are formed during the initial stages of P. tirupatiensis seedling establishment constitute also a growing shoot apex that is hereafter designated as shoot tip explant. Shoot tips, which were considered as the best source for morphogenesis (Goyal, et al, 1985; Arrilaga and Merkle, 1991) were cultured on basic medium which was free of hormones typically developed into single seedling like shoots with strong apical dominance. MS medium variously supplemented with different growth regulator combinations were used to study their effects on multiple shoot formation. The ability of these shoots to form multiple shoots and rooting was compared with that of multiple shoots obtained from the axillary buds.
Of the various growth regulator combinations tested cytokinins alone were capable of inducing **shoot proliferation from apical bud explants**. When cultured on medium containing cytokinin, axillary shoots often developed prematurely and were followed by secondary, then tertiary shoots in a proliferating cluster. Once such a cluster has developed sufficiently, it was divided into smaller clumps of shoots or separate shoots that foiled similar cluster when cultured on fresh medium. Although, luxurious shoot growth could be observed in all tested concentrations the shoot number and length varied with concentration and type of cytokinin used.

Combinations of BA with KN and BA with auxins like NAA, IBA, IAA did not form multiple shoot clusters but the formation of basal callusing was observed to be significantly higher than when the medium as supplemented with BA alone. These results are in contrast to many reports on BA promoted shoot multiplication (Khan, *et al.*, 1997). Although optimal requirement of BA-NAA used for shoot morphogenesis was varied in different plants, production of stunted shoots with short internodes at high concentration of BA alone or with NAA is common to the three plants studied. This is consistent with the published reports (Hossain, *et al.*, 1992).

Axillary shoot system is the best suited **in vitro** culture system for conservation purposes. This is due to the reduced risk of somaclonal variation (Larkin and Scowcraft, 1981). Axillary buds grown on growth regulator free medium have developed into single shoots practically without axillary shoot proliferation. These single shoots were long petioled with dark green leaves and were less curled than the normal leaves. They also necrosed rapidly especially in presence of any auxin in the medium. However, when these buds were cultured on MS medium containing BA alone or in combination with NAA, differentiation of primary rosettes with pale green, short-petioled leaves were observed. Among the several combinations of BA and NAA tested, highest number of multiple shoot rosettes were obtained in presence of BA (1.0 mg/l + NAA 0.5 mg/l). Increasing the concentration of NAA decreased the number of shoots formed and increased the basal
callus formation. As the concentration of BA was increased, up to 1 mg l⁻¹ there was marked increase in the mean number of multiple shoots per explant, which started decreasing at higher levels of BA concentration. Liu et al. (1998) have reported that the type of explant can also influence the effective concentration of growth regulator for promoting morphogenic response in vitro. The presence of NAA in the regeneration medium inhibited multiple shoot initiation regardless of BA concentration similar to the results of Valobra and James (1990).

Mass propagation of shoots obtained from repeated subculturing of explants derived from aseptic shoot cultures is yet another method practiced in many plant systems (Krishnan and Seen, 1994; Hosaki and Katahira, 1994). Multiple shoots can be subcultured repeatedly by excising the shoots from the cluster and transferring them on to fresh medium with four weeks of passage time. Large clumps of multiple shoots were separated and subcultured as small cluster for further proliferation in nutrient media supplemented with different concentration and combinations of cytokinins (BA, KIN) auxins (IAA, NAA) and GA₃. Subsequent transfer of multiple shoots to cytokinins-containing auxin-free medium resulted in adventitious regeneration. The addition of vitamins (folic acid, biotin and pantothenic acid) or complex organic substances like casein hydrolysate, yeast extract malt extract and coconut water did not significantly enhance shoot multiplication any further. Although supplementation of the medium with casein hydrolysate, yeast extract, malt extract and coconut water had shown positive responses in many other micropropagation systems, lack of significant responses in P. tiriupatiensis supports the view that for most tissue culture purposes, undefined supplements containing amino acids may be unnecessary when a correct balance of inorganic salts, particularly MS medium which has relatively high level of ammonium ions is used (George and Sherrington, 1984).

The part of a plant from which an explant is derived has major influence on direct organ formation or on the capacity of callus derived tissues to undergo morphogenesis. Under all tested combinations of growth regulators, shoots regenerated from 74% to 100% of
the stem explants were originating from first internode. Regeneration from explants originating from the second and third internodes was lower, with the latter showing the lowest regeneration efficiency. Explant taken from flowering shoots of *P.tirupatiensis* showed a gradient of flowering potential that decreases the further the explant is from the apex. Reversion from floral structures to vegetative status is fairly common and can be induced in the floral buds of some plants by appropriate cultural treatments. The rapid response of basal nodal explants over terminal nodes for shoot initiation and multiplication can be attributed to the increased cytokinin activity at the basal region besides the differential endogenous growth regulator concentration from one meristematic region to the other.

This internodal trend, as a reflection of distance from the apical meristem, was also reported in Chrysanthemum species (Lu et al., 1990; Pereira, et al., 1995). Appropriate selection of the nutrient medium is also an essential and crucial step for the success of all experimental protocols in plant tissue culture. In the present investigation MS medium has been selected for most of the experiments after a through survey of literature on related members of *Pimpinella*. It is always considered ideal to start with well known basal medium such as MS or B₅ to study a new plant system *in vitro* (Bhojwani and Razdan, 1983). Though the volume of the culture vessel is known to affect the growth of the tissue cultures (Monette, 1986) in the present investigation significant changes were not observed in the growth between different culture vessels.

Plant propagation through callus requires the induction of organogenic or embryogenic callus type from various explants (Prakash, et al., 1999). In the present study regeneration could be achieved from internodal and hypocotyl callus. Micropropagation by somatic embryogenesis refers to methods whereby embryos are produced *in vitro* from small pieces of plant tissue or individual cells. The embryos are referred to as somatic because they are derived from the somatic (vegetative) tissue, rather than from the sexual process. Both vegetative propagation and micropropagation have the potential to capture all genetic gain of highly desirable genotypes. To initiate development of somatic embryos, PEMs obtained from hypocotyl callus of *P.tirupatiensis* were transferred to basal medium
(induction medium devoid of growth regulators). Globular embryos were produced about 1 month after transfer to basal medium less than 1% of these embryos matured to a stage resembling mature zygotic embryos, with two distinct cotyledons. Although few malformed embryos were observed, the normal appearing embryos developed into seedling like plantlets following transfer to maturation medium containing TDZ (1.0 mg/l) with GA3 (1.0 mg/l).

Though conversion of somatic embryos to robust seedlings is often uneven and problematic (Janick, 1993), maturation of somatic embryo has been reported for many species of Umbellifer like Daucus carota (Fujimura and Komamine, 1975); Anethum graveolens (Seghal, 1978); foeniculum vulgare (Hunault, 1984) Pimpinella anisum (Huber, et al., 1978) Apium graveolens (Ortan, 1984) and Coriandrum sativum (Kim et al., 1996). Fasciation of the shoots on TDZ supplemented medium, has been reported in several other species such as Malus (Van Zeiuyk verkerk et al., 1986) and Rododendron (Preece and Imel, 1991), and the possible cause was attributed to the Phenyl group present in TDZ (Huetteman and Preece, 1993).

According to Krishnamurthy (1999), somatic embryos are unipolar in development and such roots are only adventitious by origin. The high frequency of somatic embryo induction in our results suggest that it might influence the endogenous level of cytokinins, auxins and abscisic acid, so as to induce the positive embryogenic response of the activated tissue (Murthy et al., 1995 and Hutchinson et al., 1996). In papaya highly embryogenic calli was obtained from sections of hypocotyl cultured on media containing 2,4-D (Fitch, 1993).

It would therefore be anticipated that the seedlings from dry somatic embryos would be more vigorous and exhibit more rapid and uniform developments than the seedlings obtained from precociously germinated somatic embryos (McKersie et al., 1989). The somatic embryo acquires sensitivity to ABA at approximately the late torpedo or early cotyledonary stages of development, just prior to precocious germination. The role of ABA sections to be common in many species including alfalfa, canola (Senaratna et al., 1991) Geranium (Marsolais et al., 1991) and Spruce (Attree et al., 1991). However, unlike conventional vegetative propagation methods, somatic embryogenesis is amenable to automation and mechanization, making it highly desirable for large-scale production of planting stock. In addition, embryogenic cultures can easily be preserved in liquid nitrogen.

Encapsulation can be considered as an important application of micropropagation, to improve the success of in vitro derived plant delivery to field or greenhouse, and to contribute to synthetic seed technology (Furamonawa et al., 1991; Mamiya and Sakamoto, 2001). Redenberg et al., (1993) reported somatic embryogenesis and encapsulation of somatic embryos. Encapsulation techniques are useful in exchange of sterile material between laboratories due to small size and relative ease in handling the structures and also in germplasm conservation (Accart et al., 1994). The in vitro developed micro shoots encapsulated with alginate (2%) has demonstrated ability for regeneration after storage at 4°C for a period of three months. However, regeneration was not observed if the duration of storage exceeded more than three months.

The encapsulated shoot buds provide mechanical protection and the required size and shape for mechanical planting, nutrients and growth promoting agents may be incorporated (Senaratna, 1992). However, the positive response of AC compared to BM in the present study can be attributed to the efficiency of AC compared to absorb toxic substances such as polyphenols produced by tissues during culture (Fridborg et al., 1978) and toxic compounds (1-5 hydroxymethyl-flural) derived from sucrose dehydrations during autoclaving (Weatherhead et al., 1978) or impurities present in the culture medium (Weatherhead et al., 1979).
The fact that encapsulated shoot buds survived after three months and were able to produce plantlets is encouraging and indicates that they can be used for short term preservation and facilitate transport to different places. Additional studies are required to better define hardening treatments and long term storage conditions of these encapsulated shoot buds. Storage of shoot buds or somatic embryos using alginate - encapsulation protocols were already reported for few species with various degree of success (Bapat and Rao, 1988; Matsumoto et al., 2001). The shoots developed in vitro (3-5 cm in length) were transferred for rooting to MS media containing half-strength major salts supplemented with IBA, NAA or IAA (0.5mg/l). Auxins pulses for rooting induction have also been reported by several workers for different species (Arrillaga et al., 1991).

In the present investigation IBA (2.0 mg/l) did not improve rooting but induced callusing at the cut ends of shoots. IAA was ineffective in inducing rooting. The result obtained with different concentrations of MS salis suggested that concentration of mineral salts in the medium play an important role in root initiation. The beneficial effect of reduced salt and sucrose concentrations during rooting phase has been discussed in several reports (Constantine, 1978; Skirvin et al., 1980). The hardened plants showed vigor and could be easily transplanted to pots containing garden soil and organic manure (1:1 ratio). The plants were eventually transferred to the field with survival rate of about 90% after 5 months each plant developed 4-6 small underground tubers. The tubers were viable and perennating in nature.

The transition in plants from vegetative state to reproductive development is of great interest though it is poorly understood (Wang et al., 2001). The cells of established shoot meristems change their pattern of development when they cease being vegetative and instead produce floral structures. In vitro flowering provides an ideal system as a means of studying the molecular biological mechanism of flowering.

In vitro flowering has also been reported in other members of the family Umbellifer (Stephen and Jayabalans, 1998). Our observation was in agreement with the data of other investigators who observed accelerated maturation sometimes resulting in very
early flowering in plants grown from in vitro plantlets (Hackett, 1987; Schwabe, 1976). In the present investigation hypocotyl callus derived multiple shoots formed flowers in vitro. It may be the result of differences in juvenility level that depends on the ontogenic age of subcultured shoots and callus (Hackett, 1985). BA and GA\textsubscript{3} have influenced flowering in vitro in the present investigation suggesting that the phytohormone concentration especially the source of cytokinin used is also an essential criteria for in vitro flowering (Meeks-Wagner et al., 1989; Wang et al., 2001).

**Genetic Diversity of Pimpinella tirupatiensis Populations**

Populations of many wild species may become extinct in a local scale, and new populations may arise on other suitable sites (Primack, 1993). Over time, a species may colonize the same habitat several times, as ephemeral habitats periodically disappear and reform. These phenomena cause variations in spatial distribution and population size (Menges, 1990). It is a general assumption in conservation biology that long-term persistence is enhanced by high genetic variability and that low levels of variability should be avoided. On the average plant species are polymorphic at 50\% of their allozyme loci. Taxonomic status, geographical range, life form, breeding system and seed dispersal mechanisms had significant effects on the levels of genetic diversity (H\textsubscript{e}) maintained by species. Endemic species had less than half the genetic diversity (H\textsubscript{e} = 0.096) of widespread species (H\textsubscript{e} = 0.202). Narrowly and regionally distributed species had intermediate values. A number of studies of rare and endangered plants have shown variation in isozyme patterns. (Young, et al., 1996; Parani and Parida, 1997).

Management of any species in situ is often difficult when there are very few individuals left that not enough recruitment is occurring to counter the mortality or when the plant is in danger of extinction through stochastic process (Haris and Gilfilder, 1992). Populations of *P. tirupatiensis* were reduced considerably in size due to habitat fragmentation and insularization. Invariably, a major threat against many endangered
species is increasing fragmentation and insularization of their habitats, which reduces gene flow among populations, and the decreased size of the populations can lead to increased genetic drift and inbreeding with in them (Ellstrand and Elam, 1993). Problems in species persistence in such small and highly isolated populations remain another major challenge in tropical conservation biology (Schellhas and Greenberg, 1996).

To select candidate populations of wild species to be given priority for conservation, genetic criteria gained from the study of molecular markers may be useful. Traditionally, diversity measures such as expected heterozygosity or percentage of polymorphic loci are considered. For conservation purposes, the uniqueness of a population (in term of its allelic composition) may be at least as important as its diversity level and priority should be given to measures of allelic richness.

A number of studies on the assessment of genetic diversity in different species using a variety of DNA markers have been reported (Lerceteau and Szmidt, 1999; Besnard and Berville, 2000 and Esselman et al., 2000). A wide variety of nucleic acid fragments are used as markers. While some occur once in a genome others are repeated. Depending on how the DNA polymorphism is studied, various types of markers are used. Restriction fragment length polymorphism (RFLPs) have been the most widely used until now though they have the advantage of being codominant and hence all genotypes in a cross can be identified. But RFLP requires large quantities of DNA, and hence plant material, and generally rely on radioactive probes, incurring safety considerations.

PCR based markers are now being used more widely. Random Amplified Polymorphic DNA (RAPD) (Williams et al., 1990) for example, require far less material because only small amounts of DNA are needed. RAPD marker-based analysis has helped to point the very low or absence of genetic polymorphism in Meconopsis paniculata and M.simplicifolia (Sulaiman and Hasnain, 1996). The technique has been used successfully for measuring diversity in plants and the patterns of variation observed closely resemble to
those obtained using more classical methods. Contrarily, no variation was detected in electrophoretic patterns of isoenzymes in populations of the endemic streamside species *Pedicularis furbishtiae* of the St. John Valley, Marine, U.S.A. (Waller, O’ Malley and Gawler, 1987). Hence, it is still of overriding importance that a sound strategy is chosen to assess genetic diversity of a population. Especially the one, which would aim to retrieve maximum available genetic diversity of a given population using optimum sample sizes. Optimum sample sizes per site can be defined as the plants required to obtain in the target population with a frequency greater than 0.05 (Marshall and Brown, 1975). Pons and Petit (1995) emphasize the necessity of sampling many populations, rather than many individuals per population, for an accurate measurement of the subdivision of gene diversity at a single locus.

Sampling of plantlets for the present investigation involved collection of mature plants during the period of August 1998 to November 1999 prior to flowering. Adequate care was taken not to include “resprouters” that had been burnt in the previous season by taking into account of the following observations like burns of the surrounding area, evidence of the scarring of the root stock, charred remains of above ground biomass etc. Due to limited distribution, coupled with the endangered status of this taxa, only a small representative samples were used in the present investigation.

The two populations chosen for the present investigation are separated by a distance of about 5 km, geographically. In the absence of any other viable populations of *P. irupatiensis* on the hills, coupled with the fact that the above two populations are the existing relic populations of this rare species, only a minimum of 11 genotypes in each population could be chosen for the present investigation. Brown (1989) proposed that a fraction of about 10% is an appropriate sample size for sampling core entries from whole collection in germplasm repositories. The calculations using this theory has lead to the result that at least 70% of the existent alleles could be drawn with 95% certainty if 10% or more of the plants were sampled from the population.
To detect all the alleles in a population the only way is to study every individual in that deme (defined as a set of individuals, such as family, stand, sub-populations, species, etc). Since this is not feasible (to study each and every individual in a deme) the solution should be to choose a sampling strategy that yields a high probability of detecting all alleles. The minimum sample size for detecting all alleles is greater under complete homozygosity than for all other forms of allelic associations. More over, it was also very difficult on this hilly locality to follow any specific sampling strategy due to its undulating nature with thorny bushes. Nevertheless adequate care was taken to select samples for the DNA polymorphic study with a distance of at least 5 meters between any two individuals. Collection of more samples from these populations would only prove detrimental to the survival of the existing populations.

A major conclusion to emerge from the last two decades of population genetic research in plants is that species differ greatly in their levels and patterns of genetic variation (Hamrick and Godt, 1990). When dealing with subdivided populations, the geography of sampling is more important than the numbers sampled with respect to preserving genetic variation (Templeton, 1993). RAPD analysis showed low DNA polymorphism in the present investigation between populations, suggesting reduced less genetic diversity, which may be threatening the survival of this endangered species in the natural endemic habitat.

**Inter population genetic diversity between Narayana Giri and Japali theertham**

Information concerning the risk of extinction of populations and the likelihood of recolonization of habitats are important variables to be considered in any model concerned with in situ conservation and management of wild populations (Gliddon and Goudet, 1994). Knowledge of natural patterns of variability is necessary if reintroductions and translocations are to stimulate natural process.
One of the more pronounced consequences of population bottlenecks is the reduction in the number of alleles maintained in the population (Nei et al., 1975). Rare alleles are especially prone to loss following bottleneck, but when populations remain small over many generations, the effects of population bottlenecks are cumulative and there is an increasing probability that common alleles will also be lost Barrett and Kohn (1991). The genotypes a, b, d, g, h, i and j have formed a single major cluster between genetic distance values of 0.07 to 0.14 suggesting the possibility of genetic homogeneity in Japali theertham populations, while in the Narayanagiri population, the number of nodes forming different clusters emerged between the values 0.13 to 0.45 showing considerable diversity.

Few of the genotypes selected in Japalitheertham were also grouped with the Narayanagiri population suggesting the possibility of gene flow between the two populations. The retention of allelic diversity sampled from a finite population will be at greater equilibrium for neutral alleles. The search is for such localized alleles whose distribution is not widespread may be important sources of disease resistance and adaptation to specific environmental conditions (Marshall and Brown, 1975). It is a general assumption in conservation biology that long-term persistence is enhanced by high genetic variability and that low levels of variability should be avoided. Very small populations (the extreme being single plants) are particularly at risk, as are populations growing at the limits of geographic or altitudinal range.

Many endangered species are genetically depauperate and unable to respond in situ, to the new selection pressure imposed by climatic changes (Bradshaw & Mc Neely, 1991). For example, low variation was detected in the endangered island endemic Malacothamnus fasciculata var nesioticus of Santa Cruz, Island, California. (Swenson et al., 1995) and in Swedish populations of the rare species Vicia Pisiformis (Gustafsson and Gustafsson, 1994). The role of organisms with less obvious ‘Keystone’ roles, including pollinating insects, mutualistic symbionts and population regulating pathogens and biocontrol agents can also have effects on the biodiversity of a site (Hawksworth et al., 1994; LaSalle and Gauld 1993).
Demographic monitoring of all life cycle stages (Davy and Jefferies, 1981) has revealed some important implications in monitoring certain rare plants. A number of studies of rare and endangered plants have variation in isozymes. (Young et al., 1996). Studies of the rare perennial herb Tephrosieris integrifolia (Senecio integrifolius) in Sweden revealed that in small populations reproduction fails through lack of insect visits, but the investigation also suggested that population size is not the only factor, the density of flowering individuals being also important (Widen, 1993). Pinus torreyana, of California, was known from two populations and electrophoretic studies have revealed no significant intrapopulation variation (Ledig, 1986). The value of data on genetic variation for conservation of rare species has gained increasing recognition (Falk and Holsinger, 1991), motivating numerous recent investigations of diversity for molecular polymorphism in such species (Arft and Ranker, 1998 and Austerlitz, 2000).

Invariably, a major threat against many endangered species is increasing fragmentation and insularization of their habitats which reduces gene flow among populations and the decreased size of the populations can lead to increased genetic drift and inbreeding with in them (Ellstrand and Elam, 1993). Problems in species persistence in such small and highly isolated fragmented systems remain another major challenge in tropical conservation biology (Schelhas and Greenberg, 1996). If a species is found as small, non-contiguous population or if it has populations inhabiting two or more very different types of habitat then the pattern of variation in the wild is more likely to be that of distinct ecotypes. In contrast common species, which throughout their geographical range are more or less continuously, distributed over many habitats, with all probability exhibit complex patterns of continuous variation.
The factors that are affecting their survival and persistence are varied. Although it is recognised that genotypes can vary in the ability to respond phenotypically to changes in the abiotic environment, the potential influence of subtle biotic factors such as microscopic symbionts on phenotypic expression has mostly been ignored. This is unfortunate because recently it has been reported that many plants harbor clandestine fungi that have major influences on host morphology and physiology (Marks and Clay, 1996).

Inter-disciplinary studies are essential to understand the precise causes for their extinction and to develop remedial measures. Human influence has a significant effect on the survival and persistence of populations. The present study reveals that it is possible to save endangered plants by developing appropriate methodologies. This study also shows that molecular techniques and in vitro techniques can aid in the conservation of endangered plants.